PHYSOSTIGMINE PROTECTION AGAINST LETHAL HYPOXIA IN MICE: ANALYSIS OF GENDER DIFFERENCE AND MECHANISM OF PROTECTION

by

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TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

Manuscript I:

Manuscript II:

Manuscript III:

Manuscript IV:

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In the name of God, Most Gracious, Most Merciful.

This thesis is dedicated to my parents:

Abdus Salam and Akhtar Jahan Saiyed

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Abstract

The purpose of the present thesis research was twofold: (1) to investigate the mechanisms by which physostigmine pretreatment significantly prolongs survival during hypoxia (5% O_2 - 95% N_2) in mice, and (2) to carry out a systematic gender comparison of physostigmine's protective effect and its mechanisms. No gender comparison had previously been done in the long history of pharmacological protection against lethal hypoxia in mice. The results of the research have been organized into four manuscripts (I-IV) in final form for submission to refereed journals.

The unique finding of a significant gender difference in physostigmine protection against hypoxia is reported in manuscript I. At the optimal physostigmine dose of 0.2 mg/kg 100% of treated females survived one hour of hypoxia, compared to 43% of males. In contrast, saline-treated controls of both sexes survived only three to four minutes. Dose-time-effect analyses of physostigmine-induced hypothermia and of brain cholinesterase inhibition showed no gender difference. The primary cause of hypoxic death was established as central respiratory failure, after which cardiac ventricular contractions persisted for several minutes, and significantly longer in females than in males.

The importance of central cholinergic activation by physostigmine was demonstrated in manuscript II, wherein atropine was shown to abolish physostigmine protection, as well as the hypothermia in both sexes. Furthermore, both hypothermia and brain cholinesterase inhibition were

dissociated from survival prolongation; thus, neostigmine produced hypothermia equivalent to that of physostigmine but survival times no different than controls; and with pilocarpine, not a cholinesterase inhibitor, the percentages of one-hour hypoxia survivors, and the gender difference were equivalent to those found with physostigmine.

Manuscript III describes the first continuous non-intrusive measurements of core temperatures during hypoxia in male and female mice, by means of peritoneally-implanted temperature sensitive transmitters. This sensitive technique revealed that physostigmine treated females have a significantly lower temperature than males at the onset of hypoxia, and this gender difference becomes even greater during hypoxia.

The role of sympathoadrenal activation in the mechanism of physostigmine protection is described in manuscript IV. Mice pretreated with the β blocking drug propranolol exhibited the same hypothermia and gender difference in one hour hypoxia survivors as mice treated with physostigmine alone. However, the α blocking drugs, phentolamine or prazosin, completely abolished physostigmine's protective effect without altering its hypothermic action. Maintenance of blood pressure, via α adrenergic activation is therefore an essential component in the mechanism of physostigmine protection.

From the thesis research presented in manuscripts I-IV the following conclusions can be drawn:

1. Prolongation of survival during hypoxia by physostigmine is significantly greater for female than for male mice.

2. The mechanisms of physostigmine protection depend upon central cholinergic activation, hence hypothermia, and centrally initiated α adrenergic activation.

3. Hypothermia alone is not a sufficient explanation for the mechanism of physostigmine protection. However, the significant gender difference in magnitude and rate of hypothermia after physostigmine and during hypoxia may be critical to the gender difference in survival.

4. Maintaining blood pressure and therefore cerebral blood flow via α adrenergic activation, together with hypothermia, seem to be the major mechanisms in physostigmine protection against hypoxia.

The Discussion considers the findings in relation to the hypotheses presented in the original thesis proposal, and it suggests directions for future research on the mechanisms and the gender difference in physostigmine protection.

Introduction

According to Webster (Webster's 3rd New International Dictionary 1986) the word hypoxia is defined as, "a deficiency of oxygen reaching the tissues of the body whether due to environmental deficiency or impaired respiratory and circulatory organs". The word hypoxia is used without reference to air, blood or organ. There is evidence that the word hypoxia was in use in 1940, but no knowledge of who introduced it (Campbell, 1982), and it is not found in the Quarterly Cumulative Index between 1934-1940, nor in the 1933 edition of the Oxford English Dictionary. The terms anoxia, ischemia and hypoxia had been used interchangeably, however it should be recognized that these are three different conditions, which nevertheless can all lead to reduced or insufficient oxygen concentration at the tissue or cellular level.

Cellular oxygen insufficiency or lack is the principal contribution to cell injury in several cerebrovascular, cardiovascular and pulmonary diseases. At the tissue level hypoxia can be defined as a reduction in oxygen availability to levels insufficient for the metabolic demand (Rehncrona and Siesjö, 1981), hence an impairment or failure of oxidation within the cell (Lambertsen, 1980). The oxygen availability to the tissue is the product of the arterial oxygen content and the blood flow to the tissue (Rehncrona and Siesjö, 1981). Thus any condition which causes reduction in the arterial concentration of oxygen or interferes with circulation will cause hypoxia.

The extensive literature on hypoxia testifies to the enormous amount of

research done in this field. However, the literature in the field also reveals that the question of gender-related effects of hypoxia has been examined relatively rarely. Almost all of the studies dealing with hypoxia have been done in male animals, so that comparative data for females is rare.

The experiments to be reported were undertaken to study the mechanism of protection by physostigmine against hypoxia in mice, and to include a systematic gender comparison. The thesis consists of four manuscripts, and the pertinent background information related to each manuscript is provided in the Introduction sections of each of the manuscripts. The present introduction to the thesis will provide broader background information applicable to all four manuscripts. Some redundancy in information should be anticipated.

Brief History of Oxygen and Hypoxia

It had been suspected for a long time that the air contains a vital substance necessary to sustain life. Ibn-An-Nafis (1210 - 1288) and Michael Servetus (1511 - 1553) independently described the formation of "vital spirit" in the lung by addition of air to the blood. Servetus states," The first thing to be considered is the substantial generation of vital spirit--- a compound of the inspired air with the most subtle portion of the blood".

This vital substance of the air was isolated between 1772 - 1774 independently by Joseph Priestley (1733 - 1804) and Carl Wilhelm Scheele (1742 - 1786). Scheele called it "fire air" and Priestley named it "dephlogisticated air". The

dephlogisticated or fire air was described as essential for combustion, respiration and for greening plants. In 1779 Antoine Laurent Lavoisier (1743 - 1794) named this "eminently respirable air" oxygine (from the Greek, meaning acid + to form). In Lavoisier's view oxygen must be present in all acids. It has been said that Priestley only isolated oxygen, whereas Lavoisier discovered it. Priestley, Scheele, and Lavoisier had all exposed animals to an oxygen deficient atmosphere, and all reached the same conclusion; that oxygen is essential for life.

Eduard Friedrich Wilhelm Pflüger (1829 - 1910) introduced the concepts of respiratory quotient and tissue respiration, and demonstrated that hypoxia stimulates breathing. Pflüger, by exposing dogs to either an oxygen deficient or a carbon dioxide rich atmosphere, concluded that both carbon dioxide excess and lack of oxygen stimulate breathing. He considered oxygen lack a respiratory stimulus far stronger and quicker in onset than carbon dioxide excess.

Man has subjected himself to hypoxia by diving under water, climbing mountains and rising to great height in balloons. The first description of "mountain sickness" was recorded by Father Acosta in 1608. Acosta was the first to point out that the condition of the air in the mountains was inappropriate for human respiration: " I am persuaded that the element of air there is so thin and delicate that it doesn't provide for human respiration which needs it to be thicker and more tempered". Paul Bert (1833 - 1886) after conducting experiments in animals (cats, guinea pigs and sparrows) and on himself in atmospheres of low oxygen or low pressure, presented the first proof that high altitude sickness is caused by a low

partial pressure of oxygen. In 1872 Bert obtained the data for the first oxygen dissociation curve. He plotted the oxygen content of blood against the barometric pressure of air, i.e., the equivalent percentage of oxygen with which the blood was equilibrated. High altitude hypoxia was the main focus of research dealing with hypoxia in 18th and early 19th century.

The term anoxemia was introduced by Bert's friend Dennis Jourdant in 1863. Joseph Barcroft (1872 - 1947), in his presidential address to the British Association for Advancement of Science in 1920, described the mechanism of anoxemia, and subdivided its causes, and also introduced the term anoxia.

Principal sources for the preceding history of oxygen and hypoxia have been: Perkins, 1964; Campbell, 1982; Astrup, 1986; Winslow and Monge, 1987.

Classification of Hypoxia

Transport of oxygen from the atmosphere to cell can be presented by the following simplified scheme.

Atmosphere --[1]--> Lung --[2]--> Blood --[3]--> Circulation --[4]--> Tissue --[5]--> Mitochondria

Interference in any step of oxygen transport can produce cellular hypoxia or anoxia, either of which can be classified on the basis of etiology. Therefore, the following classification of hypoxia is adapted from the classification originally proposed by Barcroft (1920) for "anoxemia".

1. *Hypoxic (Anoxic) Hypoxia*: In hypoxic hypoxia the oxygen availability to the

blood is reduced, which results in lower oxygen tension in the blood, and therefore the hemoglobin is not fully saturated with oxygen. The interference here occurs at steps 1 and 2 in the transport of oxygen.

Causes:

a. High altitude: Low barometric pressure reduces the partial pressure of oxygen in the atmosphere. At very high altitudes hypoxia still can occur even when breathing 100% O_2.

b. Respiratory diseases: Respiratory diseases interfere with the transport of oxygen from lung to blood, and consequently reduce oxygen content of the blood. Examples of respiratory diseases causing hypoxia include emphysema, bronchospasm, respiratory tract obstruction, pneumothorax, infection, etc., etc.

2. *Anemic (Hemic) Hypoxia*: Anemic hypoxia results from the reduction of total or functioning hemoglobin, therefore the oxygen carrying capacity of the blood is reduced, leading to a reduced concentration of oxygen in the blood. The interference here occurs at step 3 in the transport of oxygen.

Causes:

a. Decreased concentration of hemoglobin: Blood loss, deficiency state, bone marrow depression, etc.

b. Decrease in functioning hemoglobin: CO poisoning, nitrate poisoning.

3. *Stagnant Hypoxia*: The blood is saturated with oxygen, but as a result of decreased blood flow the availability of oxygen to the organ is insufficient. Hypoxia that results from a decrease in blood flow to the organ is also termed ischemic hypoxia. The interference here occurs at step 4 in the transport of oxygen. Examples of some disorders that may lead to stagnant hypoxia include shock, low cardiac output, thromboembolic diseases, etc..

4. *Histotoxic Hypoxia*: In histotoxic hypoxia the ability of the tissue to utilize molecular oxygen is impaired. The interference occurs in the respiratory enzyme systems. Examples of histotoxic hypoxia are found in poisoning by cyanide, and also in oxygen toxicity which causes inhibition of cytochrome oxidase. Histotoxic hypoxia is actually a case of impaired oxidation, rather than impaired oxygenation.

With the preceding background we can now turn to the thesis research project, which deals with hypoxic hypoxia. Therefore the remainder of this overall introduction is devoted to a more detailed examination of the research literature in hypoxic hypoxia.

Experimental Methods of Producing Hypoxic Hypoxia

In the experimental situation hypoxic hypoxia can be produced either by

reducing atmospheric pressure or reducing the oxygen concentration in the atmosphere. In earlier studies low atmospheric pressure per se was thought to be responsible for the pathophysiological effects of living at high altitude. Paul Bert (1880) was the first to demonstrate that a low partial pressure (PO_2) of oxygen was the underlying mechanism of high altitude sickness.

Oxygen concentration in the inspired atmosphere is reduced in the experimental situation by combining a low concentration of oxygen with some inert gas (usually nitrogen). Obviously, this is a far more convenient and inexpensive method for producing hypoxia than that involving low atmospheric pressure.

Factors Affecting the Physiologic and Metabolic Consequences of Hypoxia

In the present project mice were used to study the effects of acute hypoxia. Therefore, insofar as possible, the effects of acute hypoxia pertinent to the mouse and other rodents will be described. Because of the small size of mice the measurement of some physiological parameters is difficult, or impossible. However, studies have also been done in other rodent species, and the changes described below were observed mainly in rats. Examples of hypoxic effects in other species are included only if certain parameters have not been measured in the mouse or rat.

Breathing in an atmosphere of low oxygen presumably affects all organs of the body, however the central nervous system (CNS), and circulatory system

appear to be most vulnerable to hypoxia. Therefore the physiological and biochemical changes during hypoxia, to be discussed subsequently, stress the effects on the CNS and the circulatory system. As indicated earlier an important aspect of the physiologic or metabolic changes is that nearly all the studies have been done in male animals. Therefore, comparative data for the female is generally not available. There are also several factors which modify the effects of hypoxia in an organism, therefore these factors will be summarized before describing the effects of hypoxia.

1. Duration, severity and the onset of hypoxia: The duration and severity of hypoxia are the most important factors in determining the outcome of exposure to hypoxia, and responses to hypoxia may change with time. For example, in dogs the oxygen consumption sharply decreases during the first 20 minutes of hypoxia (8, 10, 16% O_2), but this is followed by a rise to prehypoxic control values in the latter half of a one hour exposure period (Hemingway 1952).

The response to acute compared to chronic hypoxia may also be very different. Acclimatization (chronic exposure) greatly increases the tolerance to hypoxia. Mice preexposed to sublethal hypoxia can subsequently survive more severe, otherwise lethal, hypoxia (Duffy et al., 1972; Minard and Grant, 1982). Also, mice preexposed to otherwise lethal hypoxia for a very short period can survive subsequent exposure to lethal hypoxia longer than unexposed mice. (Rising and Alecy, 1989) Similarly, rats preexposed to a brief period of anoxia survived a

second exposure to anoxia longer than did control animals (Dahl and Balfour, 1964). The acclimatization to hypoxia is rapid, so that in the experimental situation a gradual reduction from normal in the atmospheric oxygen concentration can be expected to have a different outcome than that in which oxygen concentration is reduced abruptly.

2. Age: Across several species younger animals are more resistant than older animals to hypoxia. The susceptibility to the lethal effect of hypoxia increases with increasing age (Fazekas et al., 1941; Himwich et al., 1941; Scremin et al., 1980).

3. Ambient temperature and humidity: The environmental temperature also influences the organism's response to hypoxia. For example, under normoxic conditions oxygen consumption is inversely related to the ambient temperature, whereas during hypoxia oxygen consumption is directly related to ambient temperature (Chevillard, 1966). The outcome of the exposure to hypoxia actually depends on both ambient and body temperature, so that body temperature and the ambient temperature are inversely related to length of survival during hypoxia (Artru and Michenfelder, 1981; Minard and Grant, 1982). Humidity of the inspired air also plays a role. Increased humidity increases the tolerance of mice to hypoxia (Phillips et al., 1947, 1950).

4. CO_2: The presence of carbon dioxide in the hypoxic atmosphere increases

the tolerance to hypoxia in guinea pigs (Gelhorn, 1936) and rats (Gelhorn, 1937). Thus the results of hypoxia experiments can be influenced by the methods of exposure (chamber size, animal size, gas flow, etc.). If the hypoxic gas mixture flow is too low expired CO_2 can accumulate, the amount depending upon the relative sizes of animal and chamber (Levine, 1960). Soda lime can be used to prevent the accumulation of CO_2.

5. Gender: Females seem to be more resistant to hypoxia than males (Britton and Kline, 1945).

Physiologic and Metabolic Effects of Hypoxia

1. Cardiovascular effects of hypoxia: The effect of hypoxia on the cardiovascular system has mainly been examined in large and/or anesthetized animals. In addition the hypoxic conditions were less severe than those used in the present project. There is only one study in which the heart rate of mice was examined under hypoxic conditions (5% O_2 - 95% N_2) similar to that used in the present project, and it was found that in mice the heart rate decreases during hypoxia (Scremin et al., 1980).

Acute hypoxia has been reported to increase the heart rate, cardiac output and blood pressure in anesthetized (Baugh et al., 1959; Daly and Scott, 1959) and conscious dogs (Kontos and Lower, 1969; Hammill et al., 1979; Rose et al., 1983). The cardiac and hemodynamic effects of hypoxia in the dog have been attributed

to the hypoxia-induced sympathoadrenal activation (Nahas et al., 1954; Kontos and Lower, 1969; Hammill et al., 1979; Rose et al., 1983).

In anesthetized rats hypoxia increases the heart rate, and decreases the blood pressure (Blood et al., 1946; Marshall and Metcalfe, 1988; Marshall and Metcalfe, 1989). The hypoxia-induced tachycardia has also been attributed to sympathoadrenal activation, whereas the fall in blood pressure is a direct vasodilatory effect of hypoxia (Marshall and Metcalfe, 1988). In conscious rats the cardiac output, heart rate, (Bullard, 1966) and blood pressure (Marshall and Metcalfe, 1990) decrease during acute hypoxia.

During hypoxia the coronary and cerebral blood flow increase, whereas no change occurs in the gastrointestinal, renal or hepatic blood flow (Durieux et al., 1992).

2. Cerebrovascular effects of hypoxia: During normoxic conditions an increase in the arterial blood pressure induces net cerebral precapillary vessel constriction and increases the cerebral vascular resistance (CVR). In contrast, a decrease in arterial blood pressure induces net cerebral precapillary vessel dilation and as a result causes a decrease in the cerebral vascular resistance. These compensatory responses tend to maintain the cerebral blood flow constant over a wide range of blood pressures. This phenomenon is called autoregulation (Fog, 1937; Fog, 1939; Scheinberg et al., 1976; Kontos, 1981; Strandgaard and Paulson, 1984).

Acute hypoxia causes cerebral vessel dilation, decreases cerebrovascular resistance and increases cerebral blood flow (Kety and Schmidt, 1948; Cohen et al., 1967; Kogure et al., 1970a; Borgström et al., 1975; Jóhannsson and Siesjö, 1975; Scheinberg et al., 1976; Smith et al., 1983; Javaheri, 1986). The cerebral vasodilation induced by carbon dioxide is augmented by hypoxia (Quint et al., 1980). Volatile anesthetics reduce the cerebral hyperemic effect of hypoxia (Durieux et al., 1992).

The exact mechanism of cerebral vasodilation during hypoxia is not clear. However, the mechanisms proposed to explain the phenomenon include the accumulation of vasoactive metabolites [e.g. H^+ (Kogure et al., 1970), K^+ (Morris, 1974; Krishner et al., 1975; Krishner et al., 1976) and adenosine (Emerson and Raymond, 1981; Winn et al., 1981; Wei and Kontos, 1983; Morii et al., 1983; Morii et al., 1987)]. Recent experimental evidence supports the hypothesis that increased brain adenosine causes the increase in cerebral blood flow during hypoxia. Brain adenosine levels increase rapidly (within 30 sec) during hypoxia and are closely correlated with changes in cerebrovascular resistance (Winn et al., 1981, 1984). Topical application of adenosine deaminase (Wei and Kontos, 1983) and intravascular theophylline (Morii et al., 1983; Morii et al., 1987) each markedly attenuate the increase in cerebral blood flow (CBF) during hypoxia.

During hypoxia cerebral autoregulation is inhibited and the cerebral blood flow becomes pressure dependent, i.e. CBF passively follows arterial pressure (Freeman, 1968; Freeman and Ingvar, 1968; Häggerdal, 1968; Kogure et al.,

1970b). Thus, depending on magnitude, reduction in blood pressure during hypoxia can reduce the reactive hyperemia, abolish it altogether or even cause cerebral blood flow to fall to ischemic values (Siesjö and Nilsson, 1971).

3. Effects of hypoxia on cerebral metabolism

a. Cerebral glucose: The cerebral glucose level decreases within 0.5-2 minutes after the start of hypoxia (4-5% O_2) exposure in mice (Broniszewska-Ardelt and Jongkind, 1971; Duffy et al., 1972; Berlet, 1975) and rats (Norberg et al., 1975). The initial changes in cerebral glucose are reversible as the hypoxia continues. Thus, cerebral glucose has been reported to be normal or above normal by 15-30 minutes of hypoxia in mice (Duffy et al., 1972) and rats (MacMillan and Siesjö, 1972a; Bachelard, et al., 1974; Norberg and Siesjö, 1975a; Petroni et al., 1984; Karcher et al., 1984). The cerebral metabolic rate of glucose therefore increases during the first 2 minutes of hypoxia, and after 15 minutes has returned to control values. In the initial period of hypoxia the glucose is used faster than it can be transported from the blood, and at later times either glucose consumption is decreased or its transport rate is increased (Duffy et al., 1972).

b. Lactate: Hypoxia increases the rate of anaerobic glycolysis (Bachelard, et al., 1974; Norberg and Siesjö, 1975a) and as a consequence the arterial and cerebral lactate level increases. The accumulation of arterial

and cerebral lactate is directly related to the severity (Siesjö and Nilsson, 1971; Macmillan and Siesjö, 1972a,b; Lewis et al., 1973b) and the duration (Norberg and Siesjö, 1975b; Norberg et al., 1975) of hypoxia. Accumulation of lactate is the earliest metabolic change observed during hypoxia. Thus the accumulation of cerebral lactate occurs at a level of hypoxia which does not cause a significant change in the labile phosphates (Siesjö and Nilsson, 1971; Duffy et al., 1972; Macmillan and Siesjö, 1972; Lewis et al., 1973b; Bachelard, et al., 1974; Norberg et al., 1975).

c. High energy phosphates: In anesthetized, artificially ventilated rats various levels of hypoxia up to 30 minutes duration did not alter the cerebral energy state as measured by levels of adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), or the energy charge potential ($ECP = \frac{[ATP] + 0.5[ADP]}{[ATP] + [ADP] + [AMP]}$) (MacMillan and Siesjö, 1971; Siesjö and Nilsson, 1971; MacMillan and Siesjö, 1972a; Lewis, et al., 1973; Bachelard, et al. 1974). The phosphocreatine (PCr) and PCr/creatine (Cr) ratio however both decrease during hypoxia (Siesjö and Nilsson, 1971; MacMillan and Siesjö, 1972b; Bachelard, et al.; 1974).

In conscious rats hypoxia of similar or lesser degree than that in anesthetized rats causes, within 20 seconds, a decrease in cerebral ATP and PCr, and an increase in cerebral ADP, AMP and creatine (Norberg et

al., 1975;). In conscious mice the results are conflicting. Berlet (1975) reported that a decrease in cerebral ATP and PCr, and increase in ADP, AMP and Cr occurs within two minutes after the start of hypoxia (5% O_2). Broniszewska-Ardelt (1971) did not observe any change in cerebral ATP two minutes after hypoxia (4% O_2). However a decrease in phosphocreatine (PCr) and increase in ADP, AMP was observed. Duffy et al (1972) did not observe any change in ATP, 15 minutes after hypoxia (4 or 5% O_2), although PCr was decreased significantly.

4. Effect of hypoxia on body temperature: In normoxic conditions most of the mammalian species are homeotherms i.e., they maintain their body temperatures within a narrow limit despite large variations in the ambient temperature (Jessen, 1985). However during hypoxia the thermoregulatory mechanism is impaired and many mammalian species behave as poikilotherms. A fall in body temperature during hypoxia has been reported in several species including dog (Gellhorn, 1937; Kottke et al., 1948; Hemingway and Nahas, 1952), guinea pig (Gellhorn and Janus, 1936; Hill, 1959), rat (Blood et al., 1949; Bhatia et al., 1969; DeFeo et al., 1970; Gordon and Fogelson, 1991) and mice (Gellhorn, 1937; Kottke, 1948; Chevillard, 1966; DeFeo et al., 1970; Gordon and Fogelson, 1991).

The magnitude of hypoxia-induced hypothermia depends on the severity and duration of hypoxia (Hemingway and Nahas, 1952) as well as

on the species, age, and the ambient temperature. Smaller sized animals have a greater fall in body temperature compared to larger animals (Gellhorn, 1937) all other conditions being equal. Younger animals drop their body temperature faster than the older (Hill, 1959) which may also be related to the size of the animal. The fall in body temperature during hypoxia is favored by a large thermal gradient. Thus, a greater hypothermic effect is observed at lower than at higher ambient temperature (Gellhorn, 1937; Hill, 1959; Gordon and Fogelson, 1991). Ambient temperature at or above the thermoneutral zone blocks the hypoxia-induced hypothermia, and at a higher ambient temperature (above 37°C), there may be an increase, instead of a decrease, in the body temperature. Rats and mice select a cooler ambient temperature during hypoxia (behavioral thermoregulation) and thereby augment the hypothermic effect of hypoxia (Gordon and Fogelson, 1991).

The decrease in body temperature during hypoxia is suggested to be the result of a decrease in oxygen consumption (Hill, 1959). Humidity in the breathing air decreases (Quimby et al., 1950) whereas the presence of carbon dioxide increases (Gellhorn, 1937) the hypothermia induced by hypoxia.

Pharmacological Protection Against Hypoxic Hypoxia (5% 0_2-95% N_2).

1. *Protection against hypoxia as a measure of cerebral protection*: It is

generally assumed that the brain is the organ functionally most sensitive to hypoxia. Survival under hypoxia is therefore loosely regarded as an index of cerebral function. This assumption is supported by earlier observations that the heart continues to beat after cessation of respiration (Himwich et al., 1941; DeHaan and Field, 1959). In addition, in man, mental function impairment occurs at a level of hypoxia which does not alter cardiovascular or renal functions (Lambertsen, 1980). Thus failure to survive hypoxia is viewed as failure of cerebral function. Hence, drugs which prolong survival are putatively drugs capable of protecting cerebral functions.

The investigation of drug protection against hypoxia is a relatively new field. In most cases the phenomenon of protection has been measured and defined in terms of prolongation of survival times but the mechanism of such protection has not been explored extensively. Hypoxic hypoxia (5% O_2 - 95% N_2) was first used by Arnfred and Secher (1962) for screening the cerebral protective effects of anesthetics in mice. Since that time the mouse hypoxia model has been used by several investigators (Appendix) for screening drugs for their putative cerebro-protective effects.

Mouse hypoxia as a model of cerebral ischemia is vulnerable to severe criticism because of the basic differences in the pathophysiology of hypoxic hypoxia and cerebral ischemia (see classification). In cerebral ischemia, both the availability of the oxygen and substrates (glucose) are reduced, and the waste products are not carried away. Hypoxic hypoxia, by contrast, results in oxygen

deprivation, but no deficit of substrate delivery or waste removal, and compensation occurs primarily by increased cerebral blood flow.

Nevertheless, the mouse hypoxia model has experimental value and relevance. Some of the pathological and biochemical changes that occur in ischemic hypoxia and hypoxic hypoxia are similar (Hoff et al., 1945; Courville, 1955), therefore this model can be used for screening the putative cerebral protective drugs. In fact many of the drugs which have been reported to have protective effects in animal models of cerebral ischemia are also protective in hypoxic hypoxia. For example, barbiturates and physostigmine are protective in both circumstances. A number of drugs have been studied using the mouse hypoxia model (Appendix) but in almost all the studies only male animals were utilized, so that data on female mice has not existed prior to the present thesis project.

2. *Cerebral protective actions of physostigmine*: In 1979 Scremin and Scremin reported the protective effect of physostigmine against hypoxia in male mice. The results were very striking, since the protection was greater than that by any previously reported drug, and protection was provided in unanesthetized freely moving animals. The protective effect of physostigmine against hypoxia in male mice was revalidated and reassessed by Scremin (1980) and by other authors (Artru and Michenfelder, 1980; Minard and Grant, 1982). In addition to hypoxic hypoxia, physostigmine also has protective effects in anemic hypoxia induced by

sodium nitrate (Gibson and Blass, 1976) stagnant hypoxia, as produced by hemorrhage (Guarini et al., 1989, 1990; Savic et al., 1992), and in cerebral ischemia (Scremin and Scremin, 1986).

The mechanism of physostigmine protection against hypoxia is not known, however Scremin (1979) has attributed it to the increase in cerebral blood flow and Artru and Michenfelder (1980) proposed hypothermia as the principal mechanism.

The original thesis proposal for this research project hypothesized that three actions of physostigmine (hypothermia, hypertension and hyperglycemia), singly or in combination, may be critical factors in protection against hypoxia:

Hypothermia: There is general agreement that hypothermia has cerebroprotective effects (Michenfelder, 1988). Hypothermia has been demonstrated to protect mice during hypoxia (Artru and Michenfelder, 1981; Minard and Grant, 1982). Hypothermia is also associated with reduced cellular function, and therefore reduced oxygen consumption (Häggerdal, 1975; Wong, 1983; Taylor, 1988). The mechanism of hypothermia protection is not known, but it has been attributed to the decrease in oxygen demand. This hypothesis has been questioned recently, by the observation that the protective efficacy of different anesthetics against cerebral ischemia does not parallel their ability to depress cerebral metabolism (Todd and Warner, 1992).

It is known that systemic administration of physostigmine produces hypothermia in rats (Varagić et al., 1971) and mice (Maayani et al., 1978). Physostigmine-induced hypothermia is mediated by central cholinergic activation.

Physostigmine is also found to reduce the oxygen consumption in rats (Varagić et al., 1971). Physostigmine induced hypothermia can be blocked by raising the ambient temperature (Varagić et al., 1971). High ambient temperature also blocks the protective effect of physostigmine against hypoxia (Artru and Michenfelder, 1980).

Hypertension: Cerebral energy metabolism is remarkably resistant to hypoxia, because of the homeostatic increase in cerebral blood flow. Cerebral levels of high energy phosphates remain unchanged in severe hypoxia as long as the blood pressure is upheld close to normal (Siesjö and Nelson, 1971; Jóhannsson and Siesjö, 1975). Cerebral tissue can extract enough oxygen to maintain the energy state of the cells, whereas a fall in blood pressure severely affects the oxygenation of the brain (Lewis et al., 1973).

Physostigmine injection is known to increase the blood pressure in anesthetized (Dirnhuber and Cullumbine, 1955) and conscious rats (Medaković and Varagić, 1957). This pressor effect of physostigmine is attributed to central adrenergic activation (Varagić and Krstić, 1966), since this effect can be blocked by the α-adrenergic blocking drug phentolamine but not by the β-adrenergic blocking drug propranolol (Medaković and Varagić, 1957; Kaul and Grewal, 1968). Physostigmine is known to increase the output of epinephrine from the adrenal glands (Stewart and Rogoff, 1921; Kaul and Grewal, 1968). However, the liberation of epinephrine does not play a significant role in the blood pressure rise after physostigmine, since the pressor effect of physostigmine is not blocked by

adrenalectomy (rather slight but significant increase) (Medaković and Varagić, 1957; Kaul and Grewal, 1968; Brezenoff, 1973). Thus physostigmine probably prevents the fall in blood pressure during hypoxia, and therefore by maintaining/increasing the cerebral blood flow protects against the development of a superimposed global cerebral ischemia during hypoxia.

Hyperglycemia: Glucose is the main substrate for energy production in the brain (Rehncrona and Siesjö, 1981). During hypoxia anaerobic glycolysis is activated, and is evidenced by increased lactate production (Bachelard et al., 1974; Norberg and Siesjö, 1975). One mol of glucose yields 2 mol of ATP by glycolysis compared to 38 mol of ATP equivalents that would form by oxidation of 1 mol of glucose. Because of this inefficient utilization of glucose the cerebral glucose level decreases rapidly (Broniszewska-Ardelt and Jongkind, 1971; Duffy et al., 1972; Berlet, 1975). Hyperglycemia increases the tolerance to anoxia/hypoxia in rats (Himwich, et al., 1941; Britton and Kline, 1945; Stafford and Weatherall, 1960) and mice (Kottke et al., 1948; Stemler et al., 1950; Holowach-Thurston et al., 1974; Masukawa and Tochino, 1989). Hyperglycemia has also been reported to increase the tolerance of mice to CO hypoxia (Koida et al., 1989) and to ischemia-hypoxia in rat (Jernigan et al., 1984).

Physostigmine dose dependently causes glycogenolysis in the liver (Varagić et al., 1967), brain (Mršulja and Terzić, 1968) and diaphragm (Mršulja et al., 1970). As a consequence of glycogenolysis the blood glucose level is increased after physostigmine injection (Hrubetz, 1937; Varagić et al., 1967). The glycogenolytic

effect of physostigmine is blocked by the β-adrenergic blocking drug propranolol but not by the α blocker phentolamine (Varagić et al., 1967; Mršulja and Terzić, 1968; Mršulja et al., 1970). The glycogenolytic effect of physostigmine is attributed to central sympathetic activation, and therefore is demonstrable only after systemic injection in vivo. In contrast, physostigmine has no glycogenolytic effect in vitro in liver (Varagić et al., 1967), brain (Mršulja, 1970) or diaphragm (Mršulja et al., 1970). Because of the need for central sympathetic activation, it is logical that neostigmine does not produce glycogenolysis in liver, brain or diaphragm after systemic administration (Varagić et al., 1967; Mršulja and Terzić, 1968; Mršulja et al., 1970). In addition, adrenalectomy does not block the glycogenolytic effect of physostigmine in liver (Varagić et al., 1967). Another important effect of physostigmine on carbohydrate metabolism is that it increases the concentration of glycogen in heart (Varagić et al., 1967; Varagić et al., 1970). There is a direct relationship between the concentration of glycogen in heart and survival under hypoxia/anoxia, so that those species with higher cardiac glycogen levels survive longer than those with lower cardiac glycogen (Dawes et al., 1959; Merrick and Meyer, 1954).

Research Objectives and the Format of the Thesis

The present thesis project was planned to explore the mechanism of physostigmine protection against hypoxia. The absence of gender comparison in the previous studies dealing with hypoxia or physostigmine therefore led to the

essential inclusion of a systematic gender comparison.

Following are the objectives originally proposed for the thesis research:

1. What is the temporal relationship between apnea and cardiovascular collapse in mice subjected to hypoxia (and is there a gender difference in this respect)?
2. Which of the major physiological and metabolic effects produced by physostigmine is/are essential for prolonging survival of mice subjected to hypoxia?
3. Does the interaction of physostigmine with hypoxia result in a significant sex difference in the physiologic and metabolic adaptations to hypoxic stress?

Most of the questions asked in the original thesis proposal are answered in the accompanying four manuscripts.

I. Manuscript I. describes a new finding i.e. a gender difference in physostigmine protection against hypoxia. This is a unique pharmacological finding, since the gender difference was observed only in one action of the drug (survival) without gender difference in hypothermia or brain cholinesterase inhibition.

II. The importance of cholinergic activation for physostigmine protection against hypoxia is described in manuscript II. In addition, it describes other

new findings of gender difference in the actions of pilocarpine and the peripheral anticholinergic drug atropine methyl nitrate.

III. A gender comparison of body temperatures during hypoxia in physostigmine and saline treated animals is described in manuscript III. This is a novel study since it represents continuous core temperature measurement during hypoxia by means of peritoneally implanted temperature-sensitive transmitters. It demonstrates that the gender difference in survival of physostigmine-treated mice is based on a significant gender difference in core temperatures during hypoxia.

IV. The importance of adrenergic activation in physostigmine protection against hypoxia is described in manuscript IV. This manuscript also describes new findings of gender difference with respect to the effects of α and β adrenergic blocking drugs.

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Manuscript I

(Submitted to Stroke, 1992)

A Significant Gender Difference in Physostigmine Protection Against Hypoxia*

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SUMMARY

Our purpose was to determine whether a gender difference existed in physostigmine protection against hypoxia (5% O_2 - 95% N_2). Male and female white mice were injected with NaCl or physostigmine (0.1, 0.2, 0.3 mg/kg) 15 min prior to hypoxia. Survival times were measured from hypoxia onset to respiratory failure. A dose-related prolongation of survival time was observed in a fraction of all physostigmine treated mice, and the remaining fraction survived the maximum hypoxia exposure time of 1 hr. The female:male ratio of 1 hr survivors was approximately 2:1 at each dose level. With 0.2 mg/kg physostigmine the ratio of 1 hr survivors was 100% of females to 43% of males, a highly significant gender difference. The magnitudes and time courses of physostigmine-induced hypothermia and brain cholinesterase inhibition were also measured, but no gender difference was found. Additionally, the temporal relationship between hypoxic respiratory and cardiac failure was analyzed. Cardiac function persists beyond respiratory failure, and ventricular contractions persist in a significantly greater percentage of females. The discussion considers possible mechanisms of physostigmine protection, including the putative major role of hypothermia. We conclude, however, that hypothermia alone is neither the sole basis for protection, nor for the gender difference.

INTRODUCTION

Arnfred and Secher[1] were the first to demonstrate that anesthetic doses of barbiturate increased the survival of mice during hypoxia. In their method the survival times of mice breathing 5% O_2 - 95% N_2 were measured from the initiation of hypoxia to complete cessation of respiration. Although both male and female mice were utilized the results were analyzed and reported without reference to gender.

In 1979 Scremin and Scremin[2] employed the mouse hypoxia model and reported that physostigmine pretreatment produced significant prolongation of survival time in hypoxic male mice. Subsequently, Artru and Michenfelder[3] showed, in male mice, that the physostigmine protective effect was nullified as ambient temperature approached body temperature. They concluded that physostigmine-induced hypothermia was therefore responsible for its protective action. The hypothermic effect of physostigmine in male mice at an ambient temperature of 25°C and its abolition at 35°C has been described previously[4,5].

The present investigation was undertaken to confirm the original work of Scremin and Scremin[2] as a starting point for further explorations of two principal questions: **1)** is there a gender difference in physostigmine's protection against hypoxia, and **2)** is hypothermia the sole, or chief, mechanism responsible for the protective effect of physostigmine? We describe herein a significant gender difference in physostigmine protection against hypoxia. Additionally, we report comparative measurements of physostigmine-induced

hypothermia and brain cholinesterase inhibition in male and female mice. Since the mouse hypoxia method[1] has long been used for screening drugs potentially protective against cerebral hypoxia-ischemia, we also present objective evidence that cardiac function continues well after respiratory failure; the immediate lethal event in this hypoxia model.

METHODS

Animals.

Male and female white Swiss-Webster mice (Bantin and Kingman) weighing 32.5 ± 0.3 g ($\bar{x}$ ± S.E. $\bar{x}$) were used in the present study. Food and water were provided ad libitum during a light dark cycle of 14/10 hours. The mice were housed in the AALAC-accredited animal care facility at this institution, and the protocol for the experiments reported here was reviewed and approved by the institutional Animal Care Committee.

Experimental hypoxia.

We replicated the method described by Scremin and Scremin (1979). Thus, a 2 liter spherical glass flask, with three stoppered openings was used as the hypoxia chamber. Gas flow inlet and outlet were made via tubing through the rubber stoppers in the lateral necks. The central neck was used for introduction of the experimental mouse, after which the opening was closed with a rubber stopper holding a thermometer to measure chamber temperature.

The humidified hypoxic gas mixture (5% O_2 - 95% N_2) flowed at a rate of 5 L/min, starting 5 min before introduction of each mouse, and then continuously throughout the duration of survival. The maximum duration of hypoxic exposure was limited to one hour, the arbitrary time limit described by Scremin and Scremin[1].

Treatment protocol and survival times.

Fifteen minutes before introduction into the hypoxia chamber mice were injected i.p. either with 0.9% NaCl or with 0.1, 0.2, or 0.3 mg/kg physostigmine hemisulphate (Sigma Chemical Co., St. Louis, MO) dissolved in 0.9% NaCl. Injection volumes were kept within the range of 4 to 8 μl/g body weight. Survival times were measured to the nearest 0.1 minute, by digital stopwatch, from the time of introduction into the chamber until complete cessation of respiration. Those animals which survived one full hour of hypoxia were removed from the chamber and observed frequently for the succeeding 48 hours. None of these one hour survivors displayed any gross evidence of neurological or behavioral deficit.

All hypoxia experiments were conducted between 0800 and 1700 at ambient room temperature 24.4 ± 0.1°C ($\bar{x}$ ± S.E.M. $\bar{x}$). The hypoxia chamber temperature measurements never differed from ambient room temperature by more than 0.7 ± 0.1°C.

Body temperature measurement.

Rectal temperatures of control and physostigmine treated normoxic animals were measured intermittently before and up-to 75 minutes after saline or physostigmine injection. Temperature was sensed by a petrolatum-lubricated thermocouple probe (Ret-3) inserted in the rectum to a depth of ¾ inch in unrestrained animals, and read from the digital display monitor (Thermalert

model TH8, Sensortek, Clifton, NJ) coupled to the probe.

Cerebral cholinesterase activity.

Mice were decapitated, the forebrain removed rapidly, rinsed and then homogenized in 1 ml ice-cold medium (120 mM NaCl, 1.2 mM KCl, 1 mM $MgCl_2$) using a 2 ml Potter Elvehjem homogenizer (at low speed). Aliquots (25 μl) of homogenate were assayed in 1 ml of the above medium containing the phosphate buffered (pH 8.0) H^+ indicator, m-nitrophenol. Upon addition of acetylcholine (ACh), with rapid stirring, the difference in indicator absorbance at 400 and 360 nm was recorded dynamically by a Hitachi dual wavelength, dual monochromator spectrophotometer. Calibrations were made with 100 μM HCl. Rates of change in absorbance at each ACh concentration (50, 100, 200, 400 and 800 μM) were measured over the first 1-2 minutes after addition and the data plotted in a Lineweaver-Burke plot to obtain V_{max} and K_m values. Cholinesterase activity was expressed as nmolH^+/min/mg protein. This new method for kinetic measurement of cholinesterase activity was developed in our laboratory and is fully described elsewhere[6].

EKG recording.

Mice were anesthetized by ketamine 200 mg/kg (i.p.). Surface electrodes were glued (Collodion U.S.P., Mavidon Corporation, Palm City, Florida) on the shaved surfaces of the ventral right arm near the axilla (-ve lead), the left side of

the chest about 2 cm below the left arm (+ve lead); and on the ventral right thigh (ground lead). The EKG complex was amplified and recorded by a Grass Polygraph (Model 79). The heart rate was calculated by counting the R waves in a 10 second segment of the record at specified time periods.

Direct observation of cardiac movements after hypoxic death.

Mice were exposed to hypoxia and immediately after the cessation of respiratory movements they were removed from the chamber and their chest was opened widely by a single scissor cut through the sternum. The auricular and ventricular contractions were observed directly for a maximum of five minutes after respiration had ceased, and the durations of auricular and ventricular contractions were then recorded.

Data analysis.

Data calculated and expressed as $\bar{x} \pm$ S.E. $\bar{x}$ (survival time in minutes, hypothermia, heart rate and ChE inhibition) were evaluated for statistical significance by analysis of variance (ANOVA) with a subsequent Bonferroni t-test[7]. Where measurements were calculated and expressed as proportions or percentages (one hour survivors, presence or absence of ventricular contractions) data were evaluated by Chi square and Fisher's exact test[7]. In all cases a P value <0.05 was considered the minimum for statistical significance.

RESULTS

Physostigmine protection in male mice.

The experiment described by Scremin and Scremin[1] was replicated exactly, and their original findings were confirmed. Table 1 shows our data and that of Scremin and Scremin for the mean survival times in groups of 14 male mice injected i.p. with 0.9% NaCl or physostigmine 0.1, 0.2 or 0.3 mg/kg 15 minutes prior to hypoxic (5% O_2 - 95% N_2) exposure. In agreement with their data analysis we also found that survival times of mice treated with physostigmine 0.2 or 0.3 mg/kg were significantly longer than those of the saline treated controls.

Scremin and Scremin[1] also observed that some fraction of the group of 14 mice at each physostigmine dose survived a full hour of hypoxia; the arbitrarily selected maximum exposure time. Comparative percentages of one hour hypoxia survivors in their experiment and in ours are exhibited in Table 1. Again, there is excellent agreement, and statistical evaluation of our data reveals a significantly greater percentage of one hour survivors at 0.2 and 0.3 mg/kg physostigmine compared to the controls.

The results are noteworthy in demonstrating that no NaCl-treated animals survive, on average, longer than 2.8 ± 0.3 minutes ($\bar{x}$ ± S.E. $\bar{x}$), and that the dose-related protective effect of physostigmine appears to be optimal at 0.2 mg/kg.

Gender difference in physostigmine prolongation of hypoxic survival time.

To our knowledge there had been no systematic comparison of physostigmine's protective efficacy against hypoxia in male and female mice. We therefore extended the experimental approach described in the preceding section to include groups of female, as well as male, mice. We found that physostigmine confers a strikingly greater degree of protection during hypoxia in female compared to male mice. Figure 1 shows that at every dose level of physostigmine the percentage of female mice surviving one hour of hypoxia is approximately two times greater than the percentage of male survivors. Thus, the one hour survival rates after 0.1, 0.2 and 0.3 mg/kg physostigmine were 30, 100 and 60% respectively for female mice, compared to 14, 43 and 28% for male mice. Despite the consistent twofold ratio in percentages of female/male one hour survivors at each physostigmine dose level a statistically significant gender difference ($P = 0.004$) obtains only at 0.2 mg/kg.

For mice surviving less than one hour of hypoxia (Fig. 2) physostigmine-treated females also exhibit dose-related prolongation of survival time, as do males, but there is no significant gender difference in this parameter. Two other points with respect to figures 1 and 2 deserve emphasis. First, it can be seen that there is no statistically significant gender difference in susceptibility to hypoxia. The survival time of 0.9% NaCl treated female mice was 2.9 ± 0.5 ($\bar{x}$ ± S.E. $\bar{x}$) minutes compared to 2.8 ± 0.3 minutes in males. None of these control animals, female or male, survived one hour of hypoxia. Second, the

optimum protective dose of physostigmine in female mice, 0.2 mg/kg, is the same as that in males.

Physostigmine-induced hypothermia.

Physostigmine is known to produce hypothermia in rodents and other species[8] yet the analysis of dose-time-effect relationships for physostigmine hypothermia in mice[9], with respect to gender has not been made previously. Figure 3 illustrates the dose-related hypothermic effect of physostigmine in normoxic male and female mice, with rectal temperature measurements made 25 minutes after injection. The hypothermia is statistically significant at 0.2 and 0.3 mg/kg physostigmine, but there is no significant male-female difference in hypothermia at any dose.

The mean (± S.E. $\bar{x}$) rectal temperatures measured in groups of normoxic male and female mice at various times before and after treatment with 0.2 mg/kg physostigmine is shown in Figure 4. Peak hypothermia occurred at 15 to 25 minutes after physostigmine administration, whereas the hypothermic effect had dissipated at 75 minutes post injection. Statistical evaluation of the data in Figure 4 revealed that there was no significant male - female difference in the degree of hypothermia at any time point. Similar rectal temperature measurements were also made in groups of normoxic male and female mice before and at various times after physostigmine doses of 0.1 and 0.3 mg/kg. Again, there was no significant gender difference in degree or time course of

hypothermia with these doses.

Rectal temperature measurements were also made in physostigmine treated (0.2 mg/kg) male (N=5) and female (N=10) mice that had survived one hour of hypoxia. In these animals the rectal probe was inserted immediately after removal of the mice from the chamber. Body temperatures recorded at that time were 27.2 ± 0.1°C and 27.9 ± 0.2°C in males and females respectively.

Physostigmine inhibition of brain cholinesterase.

Figure 5 shows the time course for inhibition of brain cholinesterase activity by 0.2 mg/kg physostigmine in male and female mice. Note that peak inhibition occurs rapidly, at least within 10 minutes after injection, followed by a slower, progressive recovery to near control values of enzyme activity at 75 minutes after physostigmine administration. At the 10 minute peak the degree of brain cholinesterase activity is seen (Fig. 5) to be within the range of thirty to forty percent of control. Although there was slightly greater peak inhibition in the brains of females compared to males the difference was not statistically significant. Similarly, the control enzyme activities in brains of NaCl-treated male and female mice (Vmax = 186 and 201 nmol/min/mg protein, respectively) were not significantly different.

Persistence of cardiac activity after cessation of respiration in hypoxic mice.

To determine objectively whether cessation of respiration (cerebral

failure) occurs prior to, simultaneously with, or after cardiac failure we examined cardiac function in two ways. First, EKG recording were made before, during and after lethal hypoxia in ketamine-anesthetized, NaCl-treated mice. Survival times for male (N=8) and female (N=6) mice under ketamine anesthesia were 3.8 ± 0.8 and 5.4 ± 3.2 minutes, respectively; not significantly different than survival times in unanesthetized NaCl-treated mice.

Table 2 summarizes the recorded heart rates before and during hypoxia, and then for 4 minutes after cessation of respiration. Table 2 shows that heart rates decay to approximately fifty percent of the immediate prelethal value between one and two minutes after respiratory failure, and are further reduced 4 minutes post mortem. There is, however, no significant gender difference in the pre- or post-lethal rates (Table 2), at least in ketamine anesthetized animals.

Since ketamine anesthesia alone halves the heart rate observed in unanesthetized mice we also performed open-chest direct visual observation of the hearts, in situ, of unanesthetized NaCl-treated mice, starting immediately (within 30 sec) after hypoxia-induced respiratory failure. In both male (N=10) and female (N=8) mice auricular contractions persisted in all cases at least up to five minutes after death. There was, however, a significant gender difference in the persistence of ventricular contractions after respiratory failure. Figure 6 shows that ventricular contractions in all 8 females were maintained throughout the first three minutes after death, whereas ventricular contractions had ceased in 7 of 10 males within two minutes after death. Even at five minutes post

mortem the incidence of persistent ventricular contractions in females is greater than that in males.

Although the EKG recordings and the direct cardiac observations do not reveal anything about overall cardiovascular status both methods clearly establish that electrical and mechanical function of the heart continues well after respiratory function has ceased.

DISCUSSION

The present investigation is the first to replicate precisely the original study of Scremin and Scremin[1]. Our results are in excellent agreement with theirs, demonstrating a dose-related significant prolongation of survival time during hypoxia in male mice. We have also confirmed the observation[1] that approximately forty percent of male mice pretreated with 0.2 mg/kg physostigmine survive one full hour of exposure to 5% O_2 - 95% N_2; a degree of hypoxia otherwise lethal to all control male mice in approximately three minutes. Furthermore, both studies concur in finding that the ceiling protective dose of physostigmine is at or near 0.2 mg/kg.

We have, however, extended the investigation of physostigmine's antihypoxic efficacy to include a comparison of male and female mice. As a consequence this is the first study to document a significant gender difference in therapeutic protection against hypoxia. Thus, at the optimal physostigmine dose level of 0.2 mg/kg 100% of female mice survive one hour of hypoxic hypoxia compared to 43% of males. Even at 0.1 and 0.3 mg/kg physostigmine the percentages of female survivors of one hour hypoxia are approximately two times greater than the respective percentages of male survivors. The data from the control, NaCl-treated, mice clearly demonstrate that these physostigmine-related gender differences are not attributable to an inherent gender difference in susceptibility to lethal hypoxia.

As an initial part of our search for the mechanism(s) underlying

physostigmine prolongation of survival during hypoxia we have also measured brain cholinesterase inhibition and body temperature in physostigmine-treated male and female mice. The peak inhibition of brain cholinesterase activity (Fig. 5), though not different in males and females, can reasonably be assumed sufficient to activate central cholinergic pathways that ultimately effect both hypothermia[9-12] and sympathoadrenal discharge[13,14]. Although physostigmine-induced hypothermia in mice has been reported previously[4,9,15] none of these studies included gender comparisons. We found a slightly greater peak hypothermic response in females compared to males, but the difference was not statistically significant.

Several lines of evidence indicate that hypothermia, a well known adaptive response to hypoxia[16] is a critical element in protection. Mice cooled by low ambient temperatures have increased survival times during hypoxia[17]. Most of the drugs that prolong survival times of mice during hypoxia are also known to induce hypothermia[18]. For example, the protective effects of pentobarbital[17], thiopental[19], and physostigmine[3] are attenuated or abolished by preventing their hypothermic action.

Since there is a direct correlation between body temperature and the rate of cerebral oxygen consumption ($CMRO_2$)[20,21,22] hypothermia reduces $CMRO_2$[23]. In addition, hypothermia causes a leftward shift in the oxyhemoglobin dissociation curve[24] which increases oxygen transport during hypoxia[25]. Oxygen solubility in blood and other body fluids is also increased by

hypothermia[26]. Overall, these consequences of hypothermia decrease oxygen demand and increase oxygen transport, thereby resulting in efficient utilization of oxygen during a time of limited availability. Carlsson et al[23] have demonstrated the integration of these elements to protect the hypoxic male rat when cooled to 27°C.

Our results show that physostigmine produces equivalent hypothermia in normoxic male and female mice. Furthermore, physostigmine-treated males and females that had survived one hour of hypoxia had equivalent rectal temperatures in the neighborhood of 27°C. It is difficult to reconcile these observations lacking a gender difference with the remarkable gender difference in physostigmine protection against hypoxia. Certainly the relatively large variances in our temperature measurements, attributable to the rectal probe method and attendant handling of the animals, may be obscuring a small but significant gender difference in the hypothermia. If we assume, however, that there is no gender difference in physostigmine-induced hypothermia there remains an alternative possibility for explaining the significantly greater antihypoxic effect of physostigmine in female mice; namely, that females have a decrease in $CMRO_2$ greater than that in males for equivalent degrees of hypothermia. We are not aware of any study which has compared the relationship between body temperature and $CMRO_2$ in male and female mice, or in any other rodent species.

There are many other actions of physostigmine that may contribute to its

protection against hypoxia, in consort with, or independently of, hypothermia. In rats, for example, physostigmine can increase systemic blood pressure[27,28] and cerebral blood flow[28], as well as cause hyperglycemia[29] and neuroendocrine effects[30,31]. The increased cerebral blood flow has also been shown to occur even when the blood pressure rise is blocked[28]. There is however, little or no evidence with respect to all these effects in the mouse; nor is there any substantive information about gender differences in these physostigmine effects in rodents.

For some barbiturates that are protective in hypoxia it is well known that their pharmacokinetics differ significantly in males and females[32,33]. In the case of physostigmine, however, it appears highly unlikely that our observed gender difference in protection against hypoxia has any basis in a gender difference in drug disposition. Some excellent studies[34,35] have provided detailed information about the pharmacokinetics of physostigmine in the male rat, but we know of no equivalent data for male or female mice. Nevertheless, we have shown a high degree of male-female concordance in the time courses of physostigmine induced hypothermia and brain cholinesterase inhibition (Figs. 4 and 5). Assuming that these time-action curves accurately reflect drug disposition there cannot be a significant difference in physostigmine pharmacokinetics between male and female mice.

Although the mouse hypoxia model[1] has been an important investigational technique for thirty years the temporal relationship between

respiratory (cerebral) failure and cardiac failure has not been well defined. The present study provides a systematic, quantitative analysis of this relationship in untreated mice. The persistence, after cessation of respiration, of both the EKG complex and the visibly contracting (not fibrillating) heart establishes respiratory failure as the immediate cause of death. We cannot of course exclude the possibility that an ineffective cardiac output contributes to the cerebral failure. Interesting inferences might be drawn from the significant gender difference in persistence of ventricular contractions in unanesthetized control mice after respiratory failure (Fig. 6). Thus, the results could be interpreted to signify a greater resistance of the female cardiovascular system to hypoxia. If this were true, the gender difference in physostigmine protection could involve physostigmine enhancement, via hypothermia or other effects, of an inherently greater cardiovascular resistance to hypoxia in females.

In conclusion, we have demonstrated a significant gender difference in the protective action of physostigmine against lethal hypoxia in mice. Hypothermia is most likely an important element in physostigmine protection, but it may not be the sole factor nor even the basis for the gender difference in protection. Other mechanisms that may contribute to the antihypoxic effect, and the gender difference, involve physostigmine-induced cholinergic and adrenergic activation. Analysis of these autonomic factors is currently in progress in our laboratory. Our present observations also establish that respiratory failure, the measured end point in the mouse hypoxia model[1], does

indeed precede cardiac failure by many minutes.

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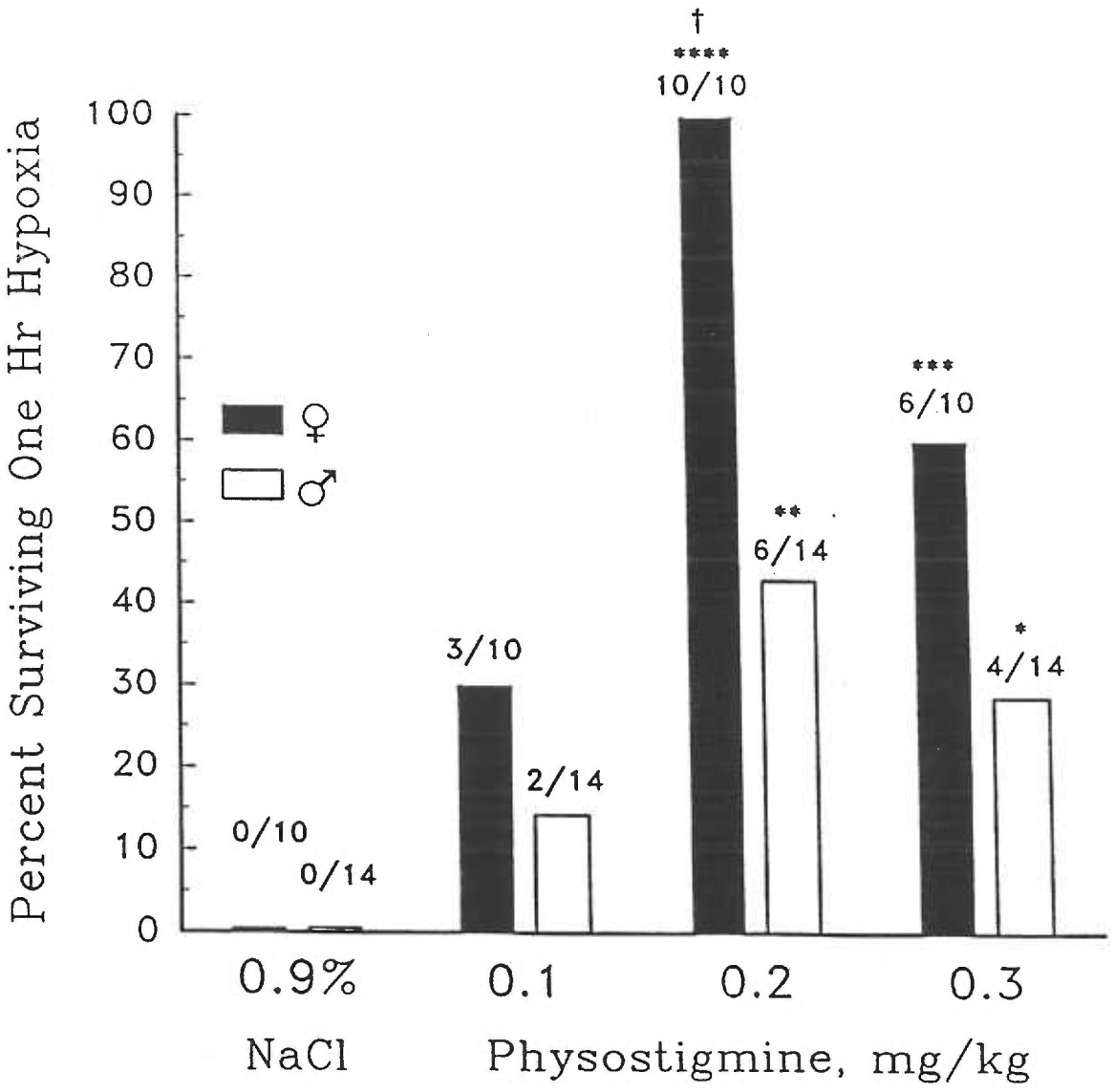
Figure 1: Gender comparison in the protective effect of physostigmine against hypoxia (5% O_2 - 95% N_2). Bar heights represent the percentages of animals in each treatment group (14 ♂, 10 ♀) surviving one hour of hypoxia. Saline or physostigmine (0.1, 0.2, 0.3 mg/kg) was injected i.p. 15 minutes before the start of hypoxia. A significant gender difference (†$p < 0.004$) is present at 0.2 mg/kg physostigmine. Significant differences vs control are: $^{*}p < 0.05$, $^{**}p < 0.007$, $^{***}p < 0.005$, $^{****}p < 0.00001$.


Percent Surviving One Hr Hypoxia
100
90
80
70
60
50
40
30
20
10
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♂
0/10
0/14
3/10
2/14
†
****
10/10
**
6/14
***
6/10
*
4/14
0.9%
NaCl
0.1
0.2
0.3
Physostigmine, mg/kg

Figure 2: Survival times, in minutes ($\bar{x}$ ± S.E. $\bar{x}$), of male and female mice dying within less than one hour of hypoxia (5% O_2 - 95% N_2). Fourteen male and ten female mice were used for each treatment group. The numbers above each bar represent the fraction of each treatment group that survived the mean time indicated on the ordinate. The remaining fraction of each group survived one hour (see figure 1). Saline or physostigmine (0.1, 0.2, 0.3 mg/kg) was injected (i.p.) 15 minutes prior to the start of hypoxia. Significant differences vs control are: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

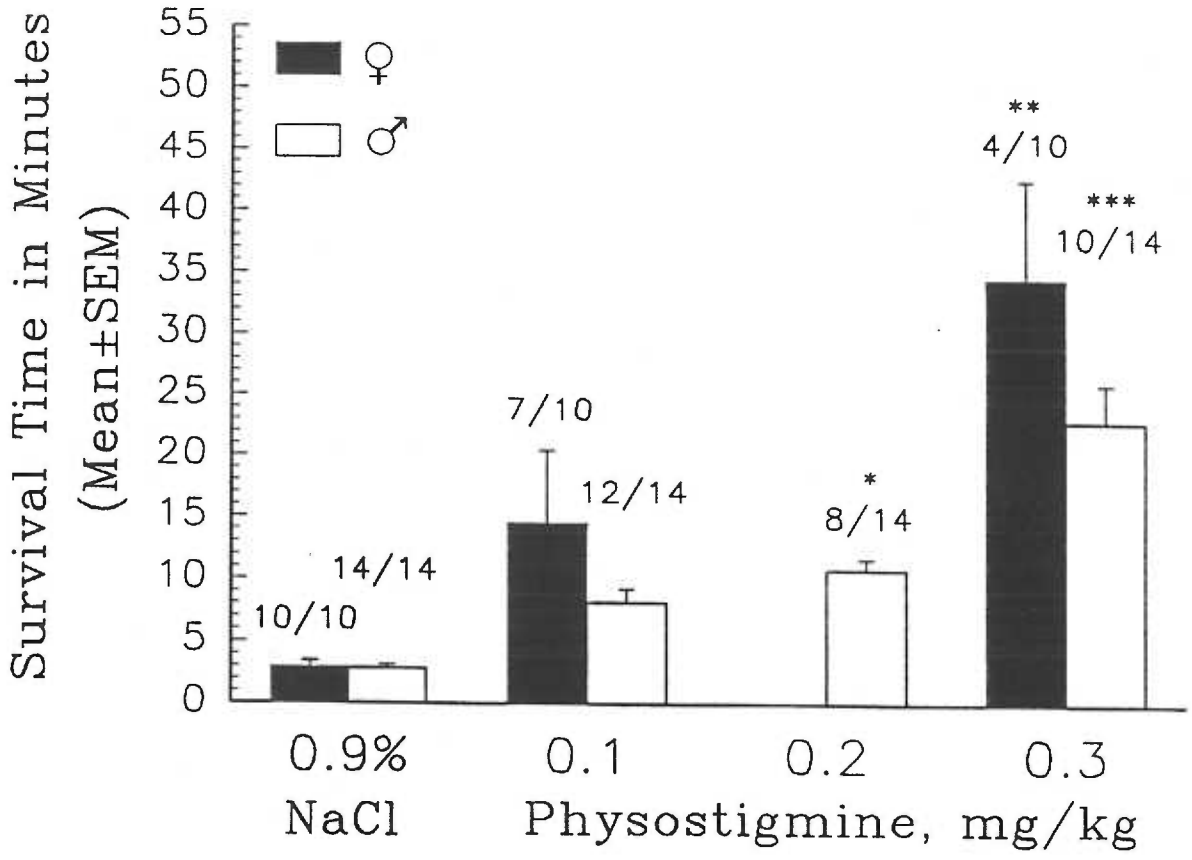
Survival Time in Minutes
(Mean±SEM)
55
50
45
40
35
30
25
20
15
10
5
0
♀
♂
10/10
14/14
7/10
12/14
*
8/14
**
4/10
***
10/14
0.9%
NaCl
0.1
0.2
0.3
Physostigmine, mg/kg

Figure 3: Dose-response curves for the hypothermic effect of physostigmine in unrestrained, normoxic male and female mice. Rectal temperatures were measured in NaCl-treated animals 25 minutes after i.p. injection of either NaCl (0.0 on abscissa) or the indicated doses of physostigmine. The number of animals for each treatment were (male, female respectively): NaCl (6,4), 0.1 mg/kg (7,7), 0.2 mg/kg (18,13) and 0.3 mg/kg (7,5). Significant difference vs control: $^{*}p < 0.001$.

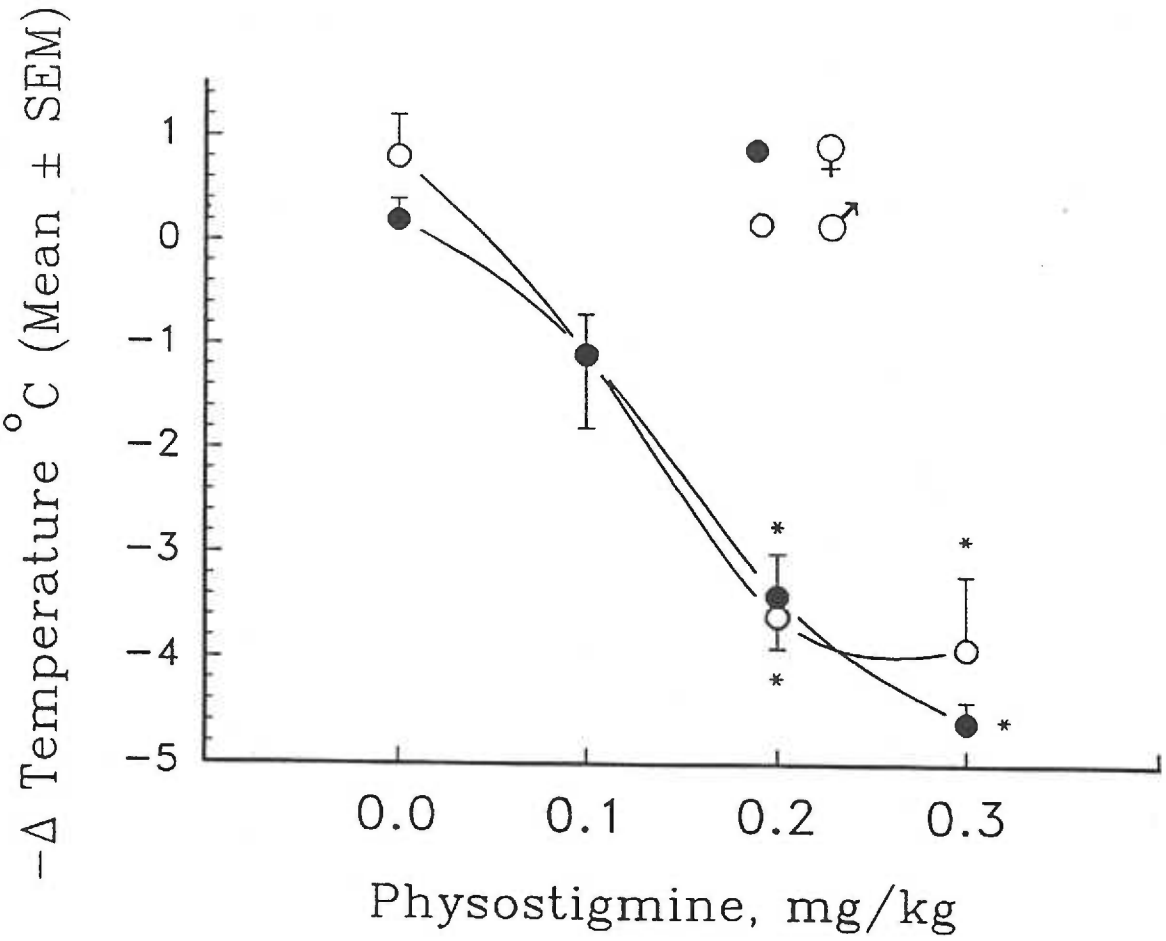
−Δ Temperature °C (Mean ± SEM)
1
0
−1
−2
−3
−4
−5
0.0
0.1
0.2
0.3
Physostigmine, mg/kg
♀
♂
*
*
*
*

Figure 4: Time course of hypothermia induced by 0.2 mg/kg physostigmine, i.p., in unrestrained normoxic male and female mice. Rectal temperature were measured intermittently before and after physostigmine injection. Each value represents the ($\bar{x}$ ± S.E. $\bar{x}$) of body temperature measurements in 16-20 male and 10-16 female mice. Significant differences from control temperatures are: $^{*}p < 0.05$, $^{**}p < 0.005$.

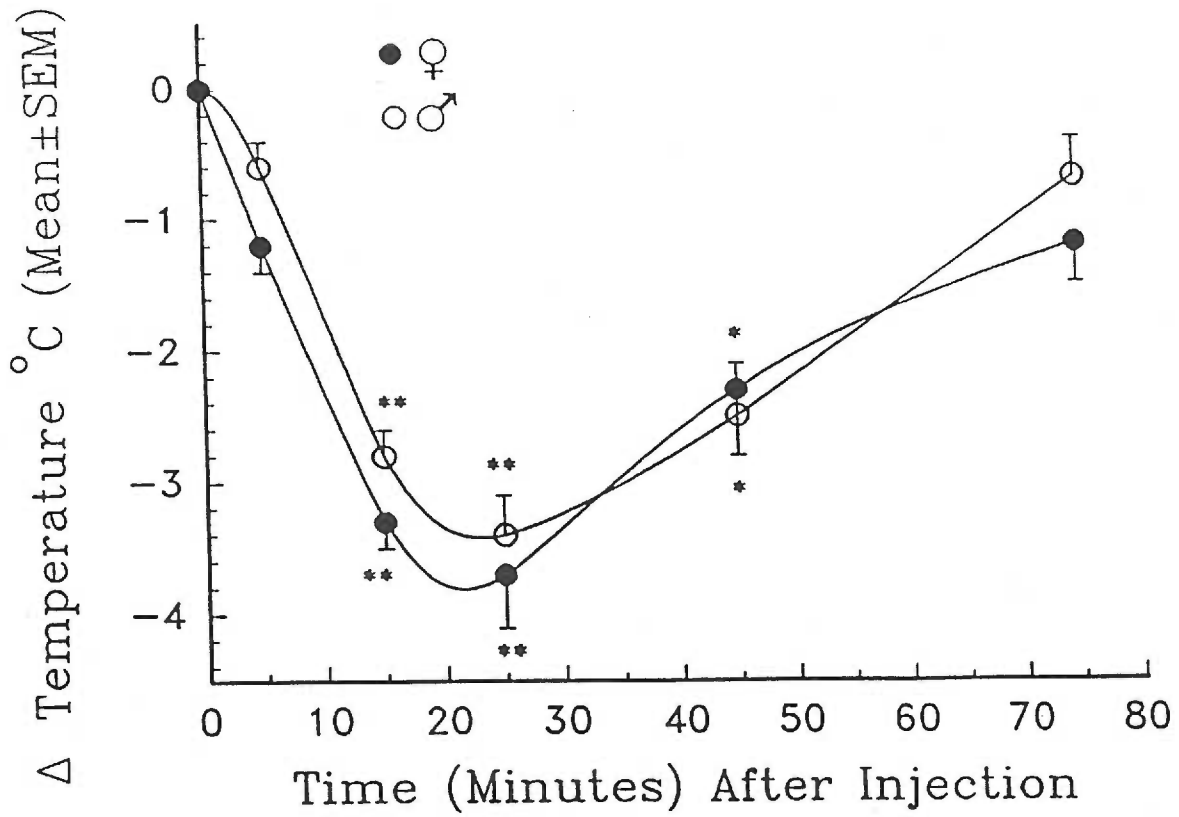
Δ Temperature °C (Mean±SEM)
0
−1
−2
−3
−4
♀
♂
**
**
**
**
*
*
0
10
20
30
40
50
60
70
80
Time (Minutes) After Injection

Figure 5: Time course of cholinesterase inhibition in male and female mouse brain, after i.p. administration of physostigmine, 0.2 mg/kg. The data ($\bar{x} \pm$ S.E. $\bar{x}$) at each time point are derived from 5 male and 5 female brains. Control Vmax, taken as 100%, was also derived form 5 animals of each sex.

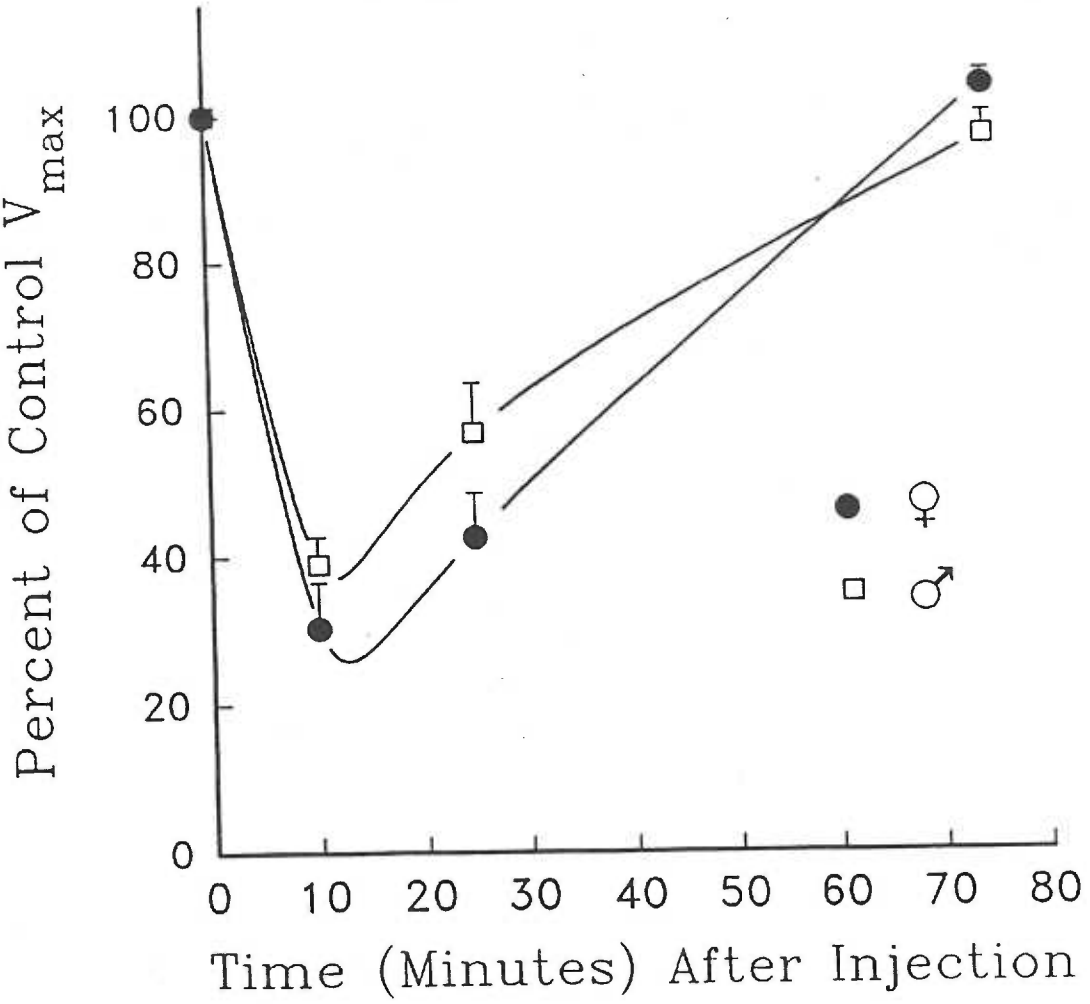
Percent of Control V_{max}
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
Time (Minutes) After Injection
♀
♂

Figure 6: Persistence of cardiac function after respiratory failure in saline-treated mice exposed to hypoxia. Ordinate: Percentage of males (N=10) and females (N=8) with ventricular contractions directly observed in situ. Abscissa: Time after apneic death from hypoxia. Note the significantly ($^{*}p < 0.005$) greater percentage of females, compared to males, with persistent ventricular contractions.

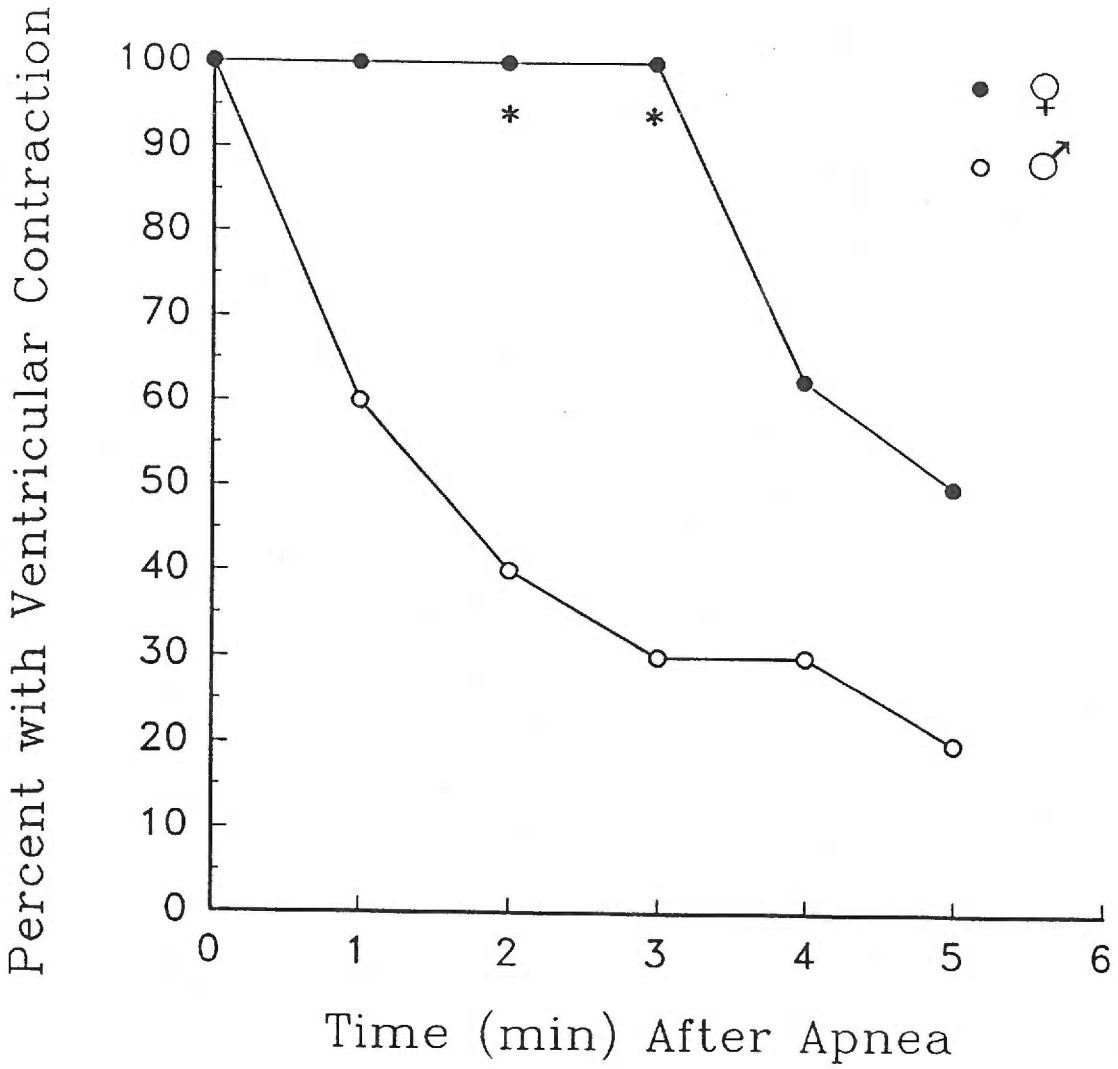
Percent with Ventricular Contraction
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
*
*
♀
♂
Time (min) After Apnea

Table 1

Comparative Results from Two Studies of Physostigmine Protection Against Hypoxia in Male Mice

Treatment	Fraction of Mice Surviving 1 Hr Hypoxia		Survival Time (min) of Mice Dying Before 1Hr ($\bar{x}$ ± S.E. $\bar{x}$) (N)	
NaCl or Physostigmine, mg/kg	Study I	Study II	Study I	Study II
NaCl	0/19	0/14	4.3 ± 0.4 (19)	2.8 ± 0.3 (14)
Phy 0.1	2/14	2/14	6.7 ± 0.9 (12)	8.1 ± 1.1 (12)
Phy 0.2	4/14†	6/14‡	13.9 ± 1.6** (10)	10.7 ± 1* (8)
Phy 0.3	4/14†	4/14†	27.6 ± 4.4*** (10)	22.8 ± 2.1*** (10)

Signicant differences from saline treated controls: † = $p<0.05$, ‡ = $p<0.005$, (Fisher's exact test)* = $p<0.05$, ** = $p<0.01$ and *** = $p<0.001$ (Bonferroni t-test). Study I, data from Scremin and Scremin[2] ; Study II, present investigation.

Table 2

Heart Rate[a] in Ketamine-Anesthetized Mice

Before, During and After Lethal Hypoxia

Gender	Pre-Hypoxia Control	After 3 min of Hypoxia	10 Sec before Apneic Death	Time after apnea (min)			
				1 min	2 min	3 min	4 min
Female (N=6)	290±22	269±27	236±38	147±13	113±12	84±10	64±12
Male (N=8)	310±7	295±11	257±19	160±16	90±7	68±7	68±6

a= all values are $\bar{X}$ ± SE $\bar{X}$. No significant gender differences at any time point.

Manuscript II

(The Journal of Pharmacology and Experimental Therapeutics, In Press, 1992)

Cholinergic and Anticholinergic Drug Effects on Survival During Hypoxia: Significant Gender Differences[1,2]

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[1]A preliminary abstract of portions of this work was presented at the XIth International Congress of Pharmacology (Riker et al., 1990).

[2]Partial support for this investigation was provided by Stroke Program Project grant NS 17493, the Medical Research Foundation of Oregon, and a Tartar Trust Fellowship to Moin Saiyed.

Running Title: Antihypoxic Drug Effects and Gender

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ABSTRACT

We examined central and peripheral components of cholinergic drug protection against hypoxia in male and female mice. Survival times were measured in groups of control and treated (i.p. injection) animals exposed to hypoxia (5% O_2 - 95% N_2). Body temperatures were also measured in separate groups of normoxic control and treated animals. Control (NaCl) animals of both sexes survived only 3.5 and 3.7 minutes of hypoxia. After physostigmine (0.2 mg/kg), however, significantly more females (82.4%) than males (40.5%) survived 35-60 minutes of hypoxia, although physostigmine hypothermia was equal in both sexes. Pilocarpine (5 mg/kg) also produced a gender difference (female > male) in survival, despite equal hypothermia. Hypothermia after neostigmine (0.2 mg/kg) was equal in males and females, yet neither sex survived longer than controls. The protective and hypothermic effects of physostigmine were blocked by atropine sulphate (5 mg/kg). In contrast, atropine methylnitrate (2 mg/kg) did not block physostigmine hypothermia in either sex, but markedly decreased physostigmine's protective effect in females. Beside the significant gender differences in physostigmine and pilocarpine protection, the results show that hypothermia alone is not responsible for protection or for the gender difference. Survival prolongation in males appears to depend solely on physostigmine's central actions. In females peripheral actions (e.g. hormone release from pituitary and ovary) may contribute to protection, and to the gender difference.

Arnfred and Secher (1962) were the first to demonstrate prolonged survival times during hypoxic hypoxia in mice pretreated with a barbiturate. Since then the hypoxic mouse survival time has been extensively used to investigate drugs that, putatively, protect against cerebral damage or failure from hypoxia and/or ischemia. Several cholinomimetic drugs including oxotremorine, arecoline and physostigmine have thus been found to prolong significantly the survival time of mice exposed to anoxia (King, 1987a,b) or severe hypoxia (Scremin and Scremin, 1979; Scremin et al., 1980; Artru and Michenfelder, 1980; Minard and Grant, 1982; Riker et al., 1990; Saiyed and Riker, In press). Although the mechanisms of protection against hypoxia remain ill-defined, the most effective drugs are also those that can induce significant hypothermia (Minard and Grant, 1982). Since hypothermia is a well-known adaptive response to stress in rodents (Wood, 1991; Gordon and Fogelson, 1991), there is a prevailing assumption that drug-induced hypothermia is primarily responsible for prolongation of survival during hypoxia in mice (Steen and Michenfelder, 1979; Artru and Michenfelder, 1980; Artru and Michenfelder, 1981; Minard and Grant, 1982).

Recently we reported (Riker et al., 1990; Saiyed and Riker, In Press) a striking gender difference in physostigmine's protective effects in severe hypoxia (5% O_2 - 95% N_2), wherein female mice are protected to a significantly greater degree than are males. This report of gender difference was unique in at least two respects. First, because the preponderance of work on antihypoxic

drug efficacy had either utilized male animals exclusively or, where females were used, had ignored gender in the analysis of results. Second, the significant gender difference in physostigmine's protection against hypoxia occurred despite the fact that physostigmine-induced hypothermia was equal in both sexes (Saiyed and Riker, In Press).

Physostigmine's ability to cross the blood-brain barrier and thus activate central cholinergic pathways (Somani and Dube, 1989), also results in a widespread sympathoadrenal discharge (Varagić and Krstić, 1966; Kaul and Grewal, 1968; Stamenović and Varagić, 1970). Consequently, the cholinergic and adrenergic systems may, independently or together, play a major mechanistic role not only in physostigmine protection against hypoxia but in the gender difference as well.

The present study extends and confirms the gender difference in physostigmine protection, and addresses the role of cholinergic systems in both the protective effect and the hypothermia. In the course of the pharmacological analysis reported here we have been able to show that pilocarpine, like physostigmine, confers significantly greater protection to female mice during hypoxia. The results demonstrate that central cholinergic activation is requisite for protection against hypoxia by cholinomimetic drugs, but that central cholinesterase inhibition is not necessary. Furthermore, a purely peripherally-induced hypothermia, i.e. by vasodilation, does not protect mice of either sex. Possible mechanisms underlying the gender difference in protection

by physostigmine and pilocarpine are discussed.

METHODS

Animals. Male and female white Swiss Webster mice (Bantin and Kingman, Fremont, CA), weighing 31.2 ± 0.2 g ($\bar{x}$ ± S.E.) were used in the present study. Food and water were provided ad libitum during a light/dark cycle of 14/10 hours. The mice (4 to 5 per shoe box) were housed in the AALAC-accredited animal care facility at this institution and were acclimatized for 1 week prior to use in experiments. The protocol for experiments reported here was reviewed and approved by the institutional Animal Care Committee.

Experimental hypoxia. A two liter spherical glass flask, with three openings was used as the hypoxic chamber. Gas flow inlet and outlet were made via tubing through the rubber stoppers in the lateral necks. The central neck was used for introduction of the experimental mouse, after which the opening was closed with a rubber stopper holding a thermometer to measure the chamber temperature.

The humidified hypoxic gas mixture (5% O_2 - 95% N_2) (Airco Medical Gases, Murray Hills, N.J.) flowed at a rate of 5 L/min, starting 5 minutes before introduction of each mouse, and then continuously throughout the duration of survival. The maximum duration of hypoxic exposure was limited to 35 minutes rather than the 60 minutes used in our previous study (Saiyed and Riker, In Press). As described in RESULTS the probability that a 35 min survivor would have survived 60 min is so great that the longer exposure is unnecessary. The

same conclusion was reached earlier by Scremin et al. (1980). Survival time was measured to the nearest 0.1 minute by a digital stopwatch, from the time of introduction into the chamber until complete cessation of respiration. All experiments were conducted between 0800 and 1700 at ambient room temperature, 23.3 ± 0.1°C ($\bar{x}$ ± S.E.). A regression analysis showed no relationship between these slight variations in ambient temperature and survival time.

Treatment protocol. For measuring the effects of treatment on hypoxic survival time, mice were injected (i.p.) with NaCl 0.9%, physostigmine 0.2 mg/kg, neostigmine 0.2 mg/kg or pilocarpine 5 mg/kg 15 minutes prior to exposure to hypoxia. Studies of cholinergic antagonism were made by injecting (i.p.) mice with saline, atropine sulphate (5 mg/kg) or atropine methylnitrate (2 mg/kg) 20 minutes prior to physostigmine (0.2 mg/kg). In these animals exposure to hypoxia was, as previously, instituted 15 min after the physostigmine injection. All drugs were dissolved in NaCl 0.9% and injection volumes were kept between 4 and 8 μl/g. All drugs were purchased from Sigma Chemical Co., St. Louis, MO.

Body temperature. Rectal temperature was measured intermittently before and up to 75 minutes after saline or drug injection under normoxic conditions at room temperature. Body temperature was sensed by a petrolatum-lubricated

thermocouple (ret-3) inserted into the rectum to a depth of ¾ inch in unrestrained animals, and read from the digital display monitor (Thermalert model TH-8, Sensortek, Clifton, NJ) coupled to the probe.

Data analysis. The body temperature measurements, in °C, were calculated and expressed as $\bar{x} \pm$ S.E. The hypoxic survival data is presented as $\bar{x} \pm$ S.E. survival time in minutes, as well as percentage of animals surviving 35 minutes of hypoxia.

The differences between the means of two experimental groups were evaluated for significance by t-test. However when more than two groups were involved in the comparison the significance of differences was evaluated by analysis of variance (ANOVA) with subsequent Bonferroni t-test (Dawson-Saunders and Trapp, 1990). For the data on the percentages of animals surviving 35 minutes of hypoxia the differences between groups were evaluated by chi-square and Fisher's exact test. In all cases a p value < 0.05 was considered the minimum for statistical significance.

RESULTS

I. Gender difference in prolongation of survival time by physostigmine during hypoxia. In a previous publication (Saiyed and Riker, In Press) we reported a significant gender difference in physostigmine protection (female > male) against hypoxia. For the present series of experiments it was necessary to establish contemporaneous control data for the effects of physostigmine alone on survival time during hypoxia. Consequently, physostigmine, 0.2 mg/kg was administered to 13 male and 14 female mice 15 min prior to hypoxia and survival times were measured. The results confirmed those reported in our previous study (Saiyed and Riker, In Press): namely, that a proportion of the treated animals survived, on average, less than 20 minutes, while the remaining proportion survived at least one hour of hypoxia. Also, as we reported previously, the percentage (71%) of female one hour survivors in the present experiments (10/14) was approximately twice that of male one hour survivors (5/13, 38%). The 4 females and 8 males that did not survive one hour had survival times ($\bar{x}$ ± S.E.), respectively, of 8.4 ± 1.7 min and 13.1 ± 3.0 min. There was no significant difference between these values, but each was significantly ($p < 0.05$) longer than the mean ± S.E. survival times of NaCl-treated male and female controls (3.5 ± 0.3 and 3.7 ± 0.4 min, respectively, where N=35 for each sex).

Figure 1 is a frequency histogram representing the combined data from our previous and present studies for prolongation of hypoxic survival by

physostigmine, 0.2 mg/kg. It is evident that survival prolongation is bimodal, consisting of a group of "short" survivors (<< 30 min) and a group of "long" survivors (at least 60 min). Furthermore, the percentage (83.3%) of female one hour survivors is significantly ($p < 0.005$) greater than that (40.7%) of male one hour survivors. On the basis of the combined mean and standard deviation derived from the present and previous studies for all physostigmine-treated male and female "short" survivors (11.2 ± 5.5 min) it is possible to predict, with 99.7% probability ($\bar{x}$ ± 3 S.D.) that the maximum "short" survival time would be 27.7 minutes. Consistent with this prediction we have not yet observed any survival times intermediate between the "short" and the "long" (one-hour) groups (see Fig. 1). Therefore, for humane as well as economic reasons we have now adopted a 35 minute, rather than one hour, maximum hypoxia exposure time, given the extremely high probability that a physostigmine treated animal surviving hypoxia for 35 minutes would survive 60 minutes of hypoxia if the experiment were continued that long. Experimental validation of this statistical prediction is described in the following section.

II. Comparative protective effects of pilocarpine, physostigmine and neostigmine.

a. Pilocarpine.

Pilocarpine, 5 mg/kg i.p., was administered to 9 female and 14 male mice 15 min prior to exposure to hypoxia. Figure 2 shows that a significantly ($p <$

0.04) greater percentage (78%, 7/9) of females compared to males (29%, 4/14) survived 35 minutes of hypoxia. Thus, like physostigmine, pilocarpine exhibits a significant gender difference in protection against hypoxia. Also like physostigmine the prolongation of survival times by pilocarpine is bimodal, so that the mean ± S.E. survival time for the 10 males surviving < 35 min was 10.7 ± 1.8 min, and for the 2 females survival times were 10.7 and 5.1 min (Fig. 3).

b. Physostigmine.

The gender difference in physostigmine protection was still evident with a 35 minute maximum hypoxia exposure. Thus 80% (8/10) of females compared to 40% (4/10) of males survived 35 min of hypoxia after physostigmine 0.2 mg/kg. Figures 2 and 3 show the combined data for this group along with the data from section I (above) and that in our previous study (Saiyed and Riker, In Press). The data in Figure 2 illustrate the highly significant ($p < 0.001$) gender difference in the combined 60 and 35 min survival percentages. For "short" (< 35 min) survival times the combined data (Fig. 3) demonstrate that there is no gender difference.

c. Neostigmine.

To examine the effects of peripheral cholinesterase inhibition, independent of brain cholinesterase inhibition, neostigmine, 0.2 mg/kg i.p., was administered to 10 female and 16 male mice 15 min prior to hypoxia. None of

the neostigmine treated females or males survived 35 minutes of hypoxia (Fig. 2). Furthermore, figure 3 shows that the mean ± S.E. survival times of the neostigmine treated females (4.4 ± 0.4 min) and males (3.8 ± 0.4 min) were not significantly different ($p > 0.3$) from those of the NaCl-treated animals. In contrast to the quiescent behavior of physostigmine treated mice, neostigmine treated mice showed slight tremor, or shivering, but no gross evidence of neuromuscular paralysis.

III. Effects of cholinergic antagonists on physostigmine protection.

a. Atropine sulphate.

Pretreatment with 5 mg/kg atropine sulphate, 20 minutes prior to 0.2 mg/kg physostigmine, abolished the protective effect of physostigmine (Table I) against hypoxia in both male and female mice. None of the 12 females and only 2 of 17 (11.8%) males treated with atropine sulphate plus physostigmine survived 35 minutes of hypoxia. This male-female difference was not statistically significant ($p > 0.4$), nor was the atropine-physostigmine 35 min survival incidence significantly different ($p > 0.1$) from the 35 min survival incidence in NaCl-treated animals. Table I also illustrates that the mean ± S.E. hypoxic survival times of the animals treated with atropine sulphate plus physostigmine were 5.7 ± 0.9 minutes for the 12 females and 4.3 ± 0.2 minutes for the 15 males, values that were not different ($p > 0.2$) from the survival times of NaCl-treated control animals. Similarly, control experiments for the effects of atropine

sulphate alone yielded survival times of 2.3 ± 0.3 (N=15) and 2.5 ± 0.4 (N=6) minutes for males and females respectively; again, not significantly different ($p > 0.05$) than the values for NaCl-treated controls.

b. Atropine methylnitrate.

The effects of peripheral cholinergic antagonism by the quaternary atropine methylnitrate were also tested. Because of the expected smaller volume of distribution of the quaternary form of atropine a dose of 2 mg/kg was chosen as the equivalent, in theoretical peak plasma concentrations, of 5 mg/kg atropine sulphate. In male mice, pretreatment with 2 mg/kg atropine methylnitrate 20 minutes prior to physostigmine 0.2 mg/kg did not alter the protective effect of physostigmine (Table I). Thus, 40% of males survived 35 minutes of hypoxia irrespective of whether the pretreatment prior to physostigmine was NaCl or atropine methylnitrate. In the remaining fraction (6/10) of atropine methylnitrate-physostigmine treated males the mean ± S.E. survival time was 11 ± 1.7 minutes. This survival time was not significantly different ($p>0.05$) from that of NaCl-physostigmine treated males (17.1 ± 3.3 min, N=6).

In female mice, however, atropine methylnitrate pretreatment (2 mg/kg) greatly attenuated ($p < 0.03$) the protective effect of physostigmine. Thus, only 20 % (2/10) of females survived 35 minutes of hypoxia compared to 80 % (8/10) of females treated with saline prior to physostigmine (Table I). The remaining 8

females treated with atropine methylnitrate-physostigmine had a mean ± S.E. survival time of 9.4 ± 3.3 minutes, not significantly different ($p > 0.05$) from that of similarly treated males or of NaCl-physostigmine treated females (Table I).

IV. Effects of cholinergic agonists and antagonists on body temperature.

All of the following observations on body temperature were made in groups of normoxic male and female mice at ambient room temperature, 23.9 ± 0.9°C ($\bar{x}$ ± S.E.), using the rectal probe technique described in METHODS.

a. Pilocarpine.

Systemic administration of pilocarpine reduced body temperature in a dose related manner, so that the peak decrease 25 min after 1, 5 and 10 mg/kg was -1.6 ± 0.4, -3.2 ± 0.2, and -4.3 ± 0.4°C respectively. The time course of pilocarpine (5 mg/kg) hypothermia in male (N=5) and female (N=5) mice (Fig. 4) was similar to that previously shown for physostigmine (Saiyed and Riker, In Press). Thus, the maximum drop in body temperature, significantly lower than control ($p < 0.001$), occured between 15 and 25 minutes after pilocarpine injection and returned near the control value at 75 minutes. There was no significant ($p > 0.05$) gender difference in the time course or in the maximum hypothermic effect of pilocarpine.

b. Neostigmine.

Figure 5 shows the time course of hypothermia induced by neostigmine, 0.2 mg/kg, i.p. in male (N=4) and female (N=5) mice. The maximum drop in body temperature occured near 25 minutes after injection, and at that time the body temperature was significantly ($p < 0.01$) lower than the pre-injection body temperature for both males and females, but there was no significant gender difference in the peak hypothermic effect of neostigmine. Although the time course of neostigmine hypothermia was longer than that after 0.2 mg/kg physostigmine (Saiyed and Riker, In Press), there was no significant difference ($p > 0.05$) between the maximum hypothermic effects of neostigmine and physostigmine at these doses.

c. Atropine sulphate.

The typical and significant hypothermic effects of 0.2 mg/kg physostigmine were abolished in both male (N=4) and female (N=4) mice treated with atropine sulphate, 5 mg/kg, 20 min prior to physostigmine. Consequently, body temperature measurements made intermittently (5 - 10 min intervals) after atropine and up to 75 min after physostigmine showed no significant deviation from pre-atropine control temperatures (Table II).

d. Atropine methylnitrate.

In contrast to atropine sulphate, atropine methylnitrate, 2 mg/kg, 20 min

prior to 0.2 mg/kg physostigmine did not alter the hypothermic effects of physostigmine in either male (N=6) or female (N=6) mice. Table II shows that 15 minutes after physostigmine the hypothermia in females was not significantly different than that in males, nor did these levels of hypothermia differ significantly from those measured at the same time point in males and females treated with NaCl 20 min prior to physostigmine. The significant and non-significant changes in body temperature described above and shown in Table II were still present in rectal temperature measurements (not shown) made 30 min after physostigmine administration.

DISCUSSION

The present findings add support to previous studies (Scremin et al., 1980; King, 1987a; Saiyed and Riker, In Press) which indicate that physostigmine protection against hypoxia reflects a primary cholinergic action of physostigmine in the central nervous system. Thus, the protective effect of physostigmine can be blocked by cholinergic antagonists which cross the blood brain barrier. King (1987a, 1987b) previously demonstrated that scopolamine was able to abolish physostigmine protection against hypoxia in male mice. Our data show that atropine sulphate antagonizes both physostigmine protection and hypothermia. We have additionally shown that atropine sulphate blocks these effects in both male and female mice.

Among the cholinergic agonists previously shown to prolong the survival times of male mice during hypoxia, oxotremorine and arecoline (King, 1987a) share the ability to cross the blood brain barrier. On the basis of the present study pilocarpine may now be added to the list of CNS-permeant cholinergic agonists that provide significant protection against hypoxia in mice. Most importantly, pilocarpine, like physostigmine (Saiyed and Riker, In Press), exhibits a significant gender difference (female > male) in its protective effect against hypoxia in mice. Consequently, the gender difference in physostigmine protection is not a drug-specific property. As is the case with many other antihypoxic drugs (Minard and Grant, 1982) pilocarpine also induces hypothermia, but the magnitude and the time course of this hypothermia was

not different in male and female mice; a finding similar to that in our previous gender comparison for physostigmine hypothermia (Saiyed and Riker, In Press).

The prolongation of hypoxia survival times by physostigmine and pilocarpine is seen to follow a bimodal distribution pattern in that there are "short" and "long" survival times, with no intermediate times. The most likely basis for this bimodal distribution can be found in the data of Minard and Grant (1982), who measured survival times of saline-treated male mice exposed to concentrations of oxygen ranging from 0 to 8 % . The resulting oxygen concentration-survival time curve was very steep between 5 and 6.5 % inspired oxygen, and a bimodal distribution of "short" and "long" survival times was observed over this range. Physostigmine or pilocarpine pretreatment of mice exposed to 5% oxygen therefore results in an apparent shift in the oxygen concentration-survival curve to the left (Fig. 6) i.e., a greater incidence of "long" survival times despite an oxygen concentration (5%) that, in NaCl-treated animals, is virtually incompatible with long survival times (Artru and Michenfelder, 1980; Minard and Grant, 1982). What we have shown for the first time in this study and in a previous one (Saiyed and Riker, In Press) is that physostigmine and pilocarpine cause an apparent leftward shift that is significantly greater for females than for males.

Hypothermia has long been considered a critical part of the adaptive, protective response to hypoxia in rodents (Gellhorn, 1937; Wood, 1991; Gordon

and Fogelson, 1991). Hypothermia, including that induced by hypoxia, reduces the rate of oxygen consumption by all tissues, including brain (Blood et al., 1949; Rosomoff and Holaday, 1954; Carlsson et al., 1976; Hagerdal et al., 1978; Dupre et al., 1987), shifts the oxyhemoglobin dissociation curve to the left (Brown and Hill, 1923; Turek et al., 1978), and increases the solubility of oxygen in blood (Christoforides and Hedley-Whyte, 1969), and in tissue and cell water (Taylor, 1988). In addition, acute hypoxia induces a greatly increased efficiency in mitochondrial oxidative phosphorylation in rodents (Mela et al., 1976; Mela, 1979), but it is unknown whether this mitochondrial change is the result of hypoxia per se or hypoxia-induced hypothermia. All of these compensatory metabolic changes are obviously in the direction of maximizing efficient utilization of a limited oxygen supply, hence will tend to prolong survival during hypoxia. Since physostigmine and pilocarpine produced equivalent hypothermia in male and female mice, yet afforded significantly greater protection to females, the mechanism of this gender difference must involve more than hypothermia alone. One possible answer might be that the hypothermia-induced decrease in the rate of cerebral oxygen consumption is greater in females compared to males, given equal hypothermia in both sexes. This possibility is untested since the bulk of studies on hypothermia-induced metabolic changes have been carried out in male animals.

Alternatively, if hypothermia-induced metabolic changes were equal in both sexes then additional factors must be sought to account for the observed

gender difference in protection with physostigmine and pilocarpine. One such factor might be a gender difference in cardiovascular susceptibility to hypoxia. We have previously reported (Saiyed and Riker, In Press) that after respiratory failure from hypoxia in both male and female mice cardiac ventricular contractions persist for several minutes in a significantly greater percentage of females compared to males. This underlying gender difference in cardiovascular resistance to hypoxia might be amplified beneficially by the centrally-mediated sympathoadrenal discharge elicited by physostigmine (Varagić and Krstić, 1966; Kaul and Grewal, 1968; Stamenović and Varagić, 1970) or pilocarpine (Levy and Ahlquist, 1962). The results obtained with neostigmine in the present study also support an important interrelationship between cardiovascular function and hypothermia in the antihypoxic effects of physostigmine and pilocarpine, and in their gender difference. Neostigmine pretreatment produced hypothermia of equal magnitude in both male and female mice. The magnitude of hypothermia was also equivalent to that following physostigmine or pilocarpine but had an even longer time course. Nevertheless, the survival times of the neostigmine-treated males and females during hypoxia were not different than those of saline-treated controls of both sexes. The failure of neostigmine to prolong survival time, despite its hypothermic action, is likely attributable to its adverse cardiovascular effects, which include vasodilation (Perlow, 1939), hypotension (Dirnhuber and Cullumbine, 1955; Medaković and Varagić, 1957; Arsura et al., 1987), and cardiac arrhythmias (Constable et al., 1990). In this

respect our results with neostigmine are like those previously reported for reserpine and chlorpromazine (Minard and Grant, 1982), both of which produce hypothermia but afforded no protection against hypoxia. Reserpine depletion of catecholamines and chlorpromazine adrenergic blockade would also result in adverse effects on cardiovascular function during hypoxia. Finally, insofar as neostigmine's failure to prolong survival, the mechanism of neostigmine-induced hypothermia may have had a bearing on the survival outcome compared to that of physostigmine and pilocarpine. The latter two drugs produce hypothermia principally by a centrally-mediated reduction of heat production (Friedman and Jaffe, 1969; Varagić et al., 1971; Lin et al., 1980). Neostigmine induced hypothermia, unlike that produced by physostigmine or pilocarpine, occurs as a result of heat loss from vasodilation and is not blocked by atropine (unpublished observations). It would be interesting to know whether the magnitude of hypothermic reduction in cerebral oxygen consumption depends on the mechanism of hypothermia, i.e. heat loss versus decreased heat production.

An unexpected finding was the attenuation by atropine methylnitrate of physostigmine protection exclusively in female mice. This gender difference in the cholinergic blocking effects of a quaternary amine has no immediate explanation, but at least two possibilities deserve consideration. First is the possibility of a gender difference in blood brain barrier permeability to atropine methylnitrate. The evidence that this drug does not cross the blood brain

barrier to any appreciable extent is derived from studies performed in male animals (Brezenoff et al., 1988), or in those where gender was not specified (Herz et al., 1965). The fact that physostigmine hypothermia was not attenuated by atropine methylnitrate in normoxic male and female mice does not necessarily exclude a gender difference in blood brain barrier permeability during hypoxia. Hypoxia itself can increase the permeability of the blood brain barrier (Dux et al., 1984), but it is not known whether the degree of disruption by hypoxia is gender dependent. A gender difference in blood brain barrier permeability has been demonstrated in the rat with fluorescein, which was accumulated to a greater degree in the brains of females compared to males (Martinez and Koda, 1988). Thus an inherent gender difference in blood brain barrier permeability, further exaggerated by hypoxia, might allow for greater accumulation of atropine methylnitrate in the brains of females, thereby partially blocking the central protective actions of physostigmine. Support for this supposition would require knowing whether the hypothermia during hypoxia in physostigmine-treated females is also attenuated by atropine methylnitrate.

Alternatively, atropine methylnitrate attenuation of physostigmine protection in the female suggests that peripheral, cholinergically activated hormonal mechanisms might be involved in the gender difference in physostigmine (and pilocarpine) protection. Specifically, both the pituitary and the ovary are outside the blood brain barrier, hence susceptible to cholinergic activation as well as block by atropine methylnitrate. The pituitary gland has

cholinergic receptors, (Avissar et al., 1981; Labella and Shin, 1968), as well as the cholinergic enzyme markers acetylcholinesterase and choline acetyltransferase (Gallardo et al., 1977; Lederis and Livingston, 1969). In the female therefore physostigmine or pilocarpine-induced release of follicle stimulating hormone (FSH), estrogen and oxytocin could play an important role in the significantly greater incidence of female survival during hypoxia. Block of the cholinergically mediated sex specific hormonal release would thus abolish the gender difference.

Cholinergic agonists, including acetylcholine, are known to stimulate FSH release from the pituitary both in vitro and vivo (Fiorindo et al., 1975; Hall and Meites, 1982). Physostigmine and pilocarpine should therefore both be capable of causing FSH release from the pituitary. The resulting increase in estrogen release from the ovary could be one factor in the gender difference in protection since estrogen is known to have a protective effect against hypoxia (Davis and Jones, 1943). Physostigmine and pilocarpine effects on the pituitary might also act synergically with their direct cholinergic effects on the ovary. Acetylcholine can stimulate the release of ovarian oxytocin (Heap et al., 1989), and the plasma levels of oxytocin are known to rise under different types of stress (Gibbs, 1984; Nussey et al., 1988; Jenkins and Nussey, 1991), including hemorrhage (Dunning et al., 1985). Such stress-related increases are greater in females than in males (Williams et al., 1985). Oxytocin's efficacy in increasing blood pressure and cardiac output (Petty et al., 1985) could enhance protection

during hypoxic stress. In summary, the pituitary and ovary are readily accessible to, and activatable by, cholinergic agonists. Consequent release of the sex-specific hormones FSH, estrogen and oxytocin could thus account for the gender difference in pilocarpine and physostigmine protection against hypoxia. Cholinergic antagonists that do not cross the blood brain barrier would easily block the hormone release from these sites outside the barrier, thereby abolishing the possible basis of gender difference in protection.

In pursuing the mechanisms responsible for physostigmine and pilocarpine protection against hypoxia, and for the gender difference, it is reasonable to maintain the assumption that hypothermia is a major, but not the sole, factor in prolonging survival. To date we have only measured drug-induced hypothermia in normoxic animals, whereas the critical question is whether a gender difference exists in the rate or degree of hypothermia during hypoxia in drug-treated animals. With respect to factors other than hypothermia it would also be essential to know whether there is a gender difference in the magnitude of peripheral sympathoadrenal discharge initiated centrally by both physostigmine and pilocarpine. The absence of specific data for both the hypothermic and adrenergic questions underscores a more widespread paucity of pharmacological information concerning gender comparison.

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Table I: Effects of tertiary and quaternary atropine on physostigmine prolongation of hypoxic survival time.

Pretreatment	*Treatment*	♂	♀	*Gender Difference*
A. Survival time[1] (min) of mice surviving < 35 min				
--	NaCl	3.5 ± 0.3 (35)	3.7 ± 0.4 (35)	N.S. $p > 0.6$
NaCl or None	Physostigmine 0.2 mg/kg	13.3 ± 1.5*** (22)	9.9 ± 1.4* (6)	N.S. $p > 0.2$
Atropine Sulphate 5 mg/kg	Physostigmine 0.2 mg/kg	4.3 ± 1 (15)	5.7 ± 1.7 (11)	N.S. $p > 0.4$
Atropine Methylnitrate 2 mg/kg	Physostigmine 0.2 mg/kg	11 ± 1.7** (6)	9.4 ± 2.0* (8)	N.S. $p > 0.5$
B. Percent of mice surviving 35 min hypoxia				
--	NaCl	0 (0/35)	12.5 (5/40)	N.S. $p > 0.08$
NaCl or None	Physostigmine 0.2 mg/kg	40.5† (15/37)	82.4†† (28/34)	$p < 0.001$
Atropine Sulphate 5 mg/kg	Physostigmine 0.2 mg/kg	11.7 (2/17)	0 (0/12)	N.S. $p > 0.4$
Atropine Methylnitrate 2 mg/kg	Physostigmine 0.2 mg/kg	40 (4/10)	20 (2/10)	N.S. $p > 0.6$

[1]Values in A are $\bar{x}$ ± S.E. Numbers in parentheses are numbers of animals.

Significance, treatment vs control (NaCl 0.9%) in the same sex: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, †$p < 0.00005$, ††$p < 0.00001$.

Table II: Effects of pretreatment with tertiary and quaternary atropine on the hypothermic effect of physostigmine.

Pretreatment	*Treatment*	*Body temperatures (T)[a] and differences from NaCl control (Δ) 15 min after treatment*				*Gender Difference*
		Males		*Females*		
		T	*Δ*	*T*	*Δ*	
---	NaCl	38.4 ± 0.2 (6)	---	38.5 ± 0.1 (6)	---	N.S. $p > 0.6$
NaCl	Physostigmine 0.2 mg/kg	35.7 ± 0.2*** (6)	-2.7	35.1 ± 0.3*** (6)	-3.4	N.S. $p > 0.08$
Atropine Methylnitrate 2 mg/kg	Physostigmine 0.2 mg/kg	35.8 ± 0.3***	-2.6	35.5 ± 0.3*** (6)	-3.0	N.S. $p > 0.4$
Atropine Sulphate 5 mg/kg	Physostigmine 0.2 mg/kg	37.0 ± 0.3N.S. (4)	-1.4	37.1 ± 0.2N.S. (4)	-1.4	N.S. $p > 0.7$

[a]Temperature ($\bar{x}$ ± S.E.) and differences are in°C. Numbers in parentheses are numbers of animals. ***$p < 0.001$

Figure 1: Frequency histogram for the distribution of survival times during hypoxia (5% O_2 - 95% N_2) in physostigmine (0.2 mg/kg) treated mice. Bar heights represent the percentages of female and male mice that survived the time intervals shown on the abscissa. Note the sharply bimodal distribution, hence the absence of intermediate survival times between 30 and 60 min.

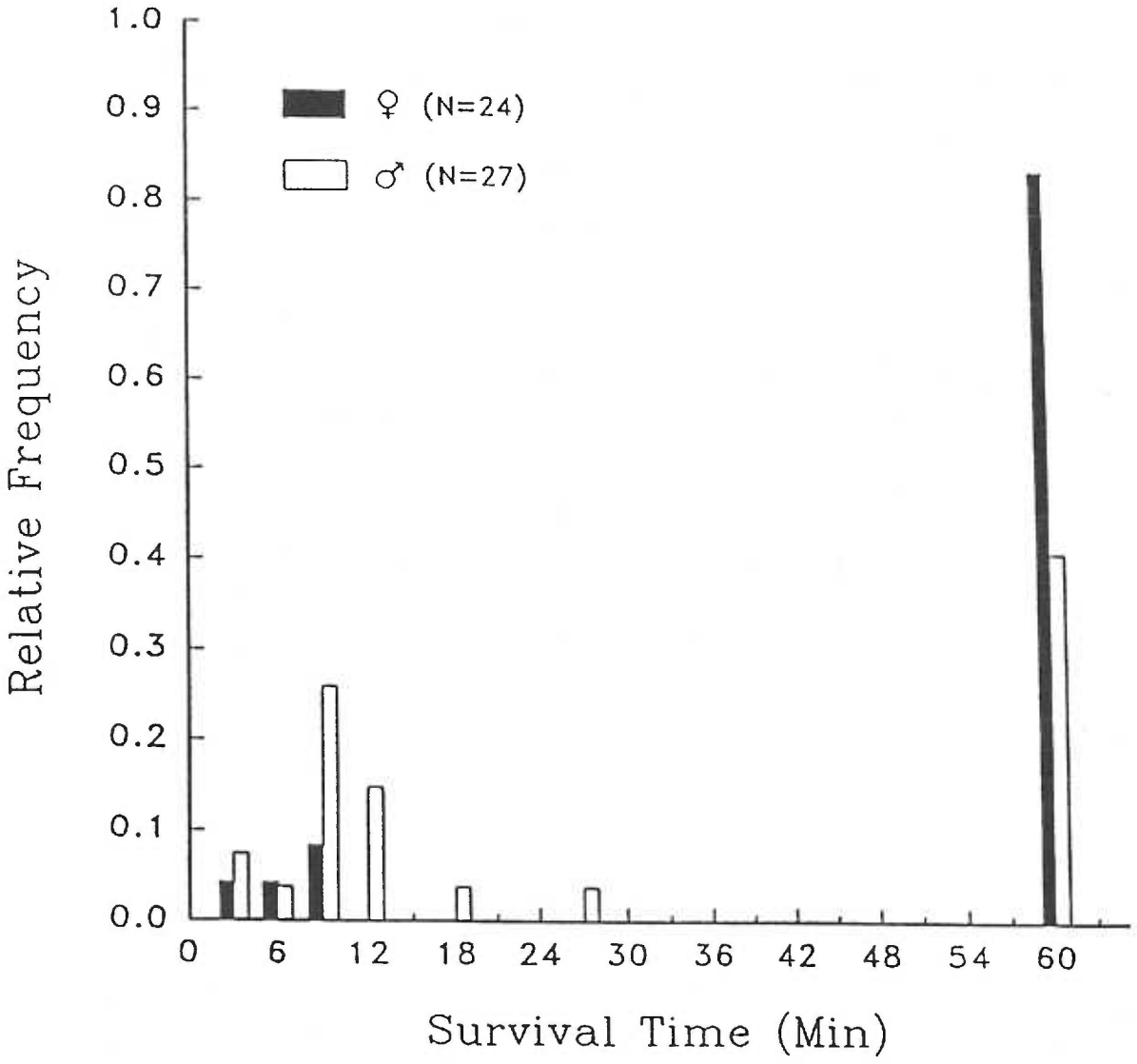

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
♀ (N=24)
♂ (N=27)
Relative Frequency
0
6
12
18
24
30
36
42
48
54
60
Survival Time (Min)

Figure 2: Percentages of female and male mice in different treatment groups surviving 35-60 minutes of hypoxia (5% O_2 - 95% N_2). Injections of NaCl, 0.2 mg/kg physostigmine (Physo), 5 mg/kg pilocarpine (Pilo) or 0.2 mg/kg neostigmine (Neo) were made 15 min before the start of hypoxia. Significant gender differences existed with physostigmine (a), $p < 0.0005$, and pilocarpine (b), $p < 0.05$, but not with NaCl ($p > 0.05$). Significant differences versus control (NaCl) were: $^{*}p < 0.005$; $^{**}p < 0.001$; $^{***}p < 0.00005$; † $p < 0.00001$. Note that no neostigmine-treated mice survived to 35 minutes.

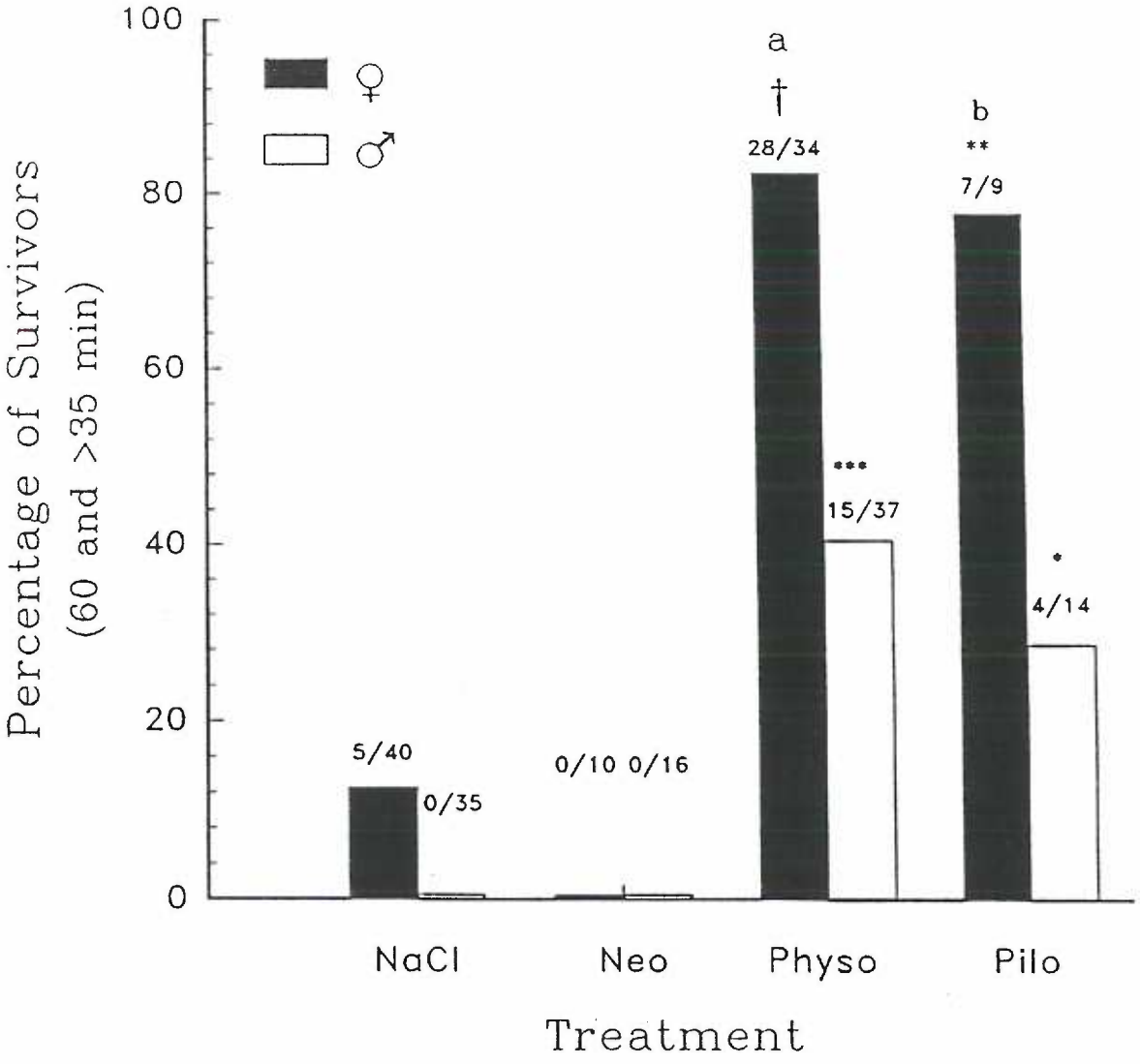

Percentage of Survivors
(60 and >35 min)
100
80
60
40
20
0
♀
♂
a
†
28/34
***
15/37
b
**
7/9
*
4/14
5/40
0/35
0/10 0/16
NaCl
Neo
Physo
Pilo
Treatment

Figure 3: Survival times, in minutes ($\bar{x}$ ± S.E.), of male and female mice in different treatment groups surviving less than 35 minutes of hypoxia (5% O_2 - 95% N_2). The number above each bar represents the fraction of each treatment group that survived the mean time indicated on the ordinate. The remaining fraction of each group survived 35 minutes or longer (see Fig. 2). Saline or drug doses and the injection protocol were the same as described in the legend to Fig. 2. No significant gender difference is present in any treatment group. Significant differences versus control (NaCl) were: $^{**}p < 0.001$.

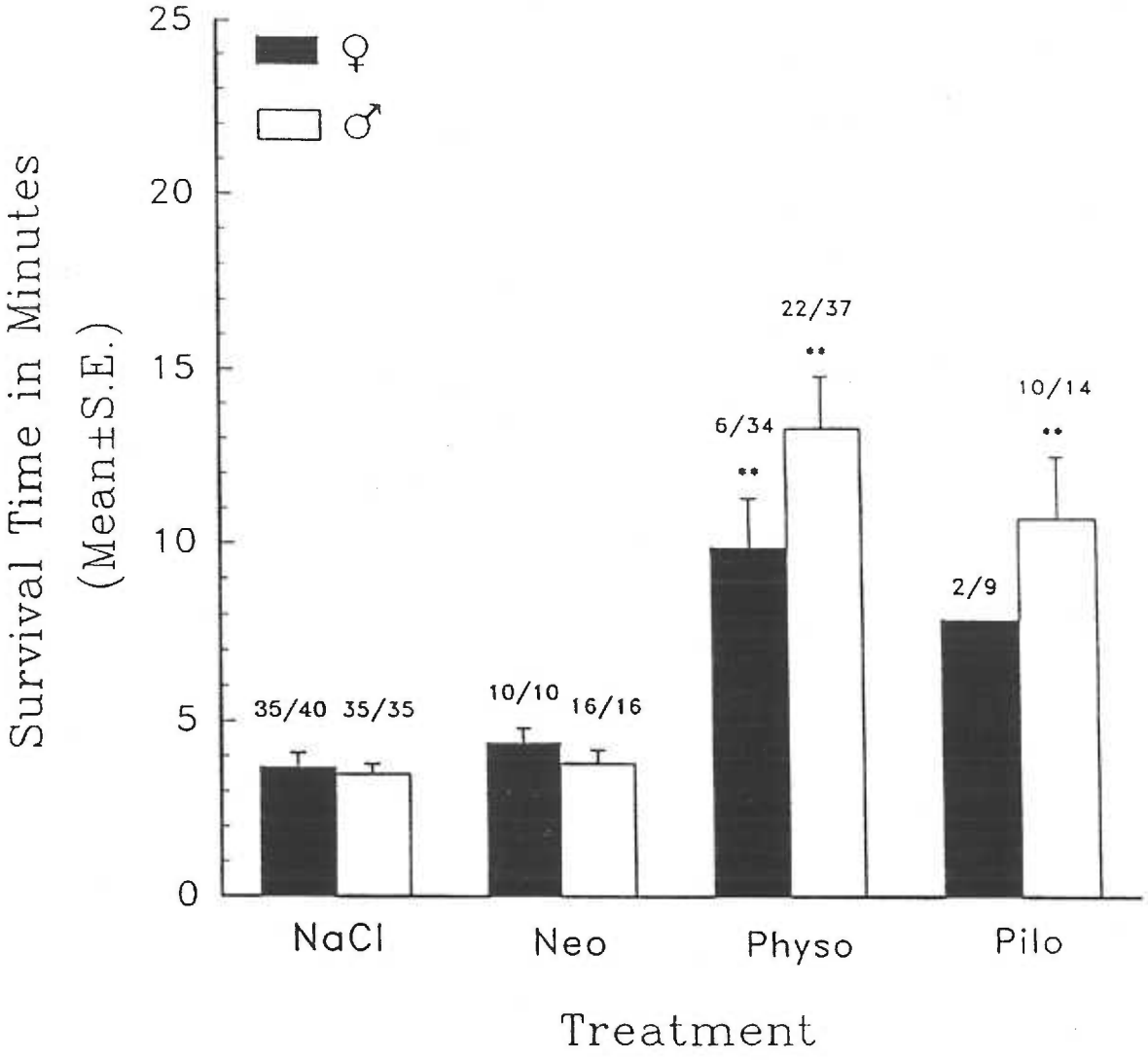

Survival Time in Minutes
(Mean±S.E.)
25
20
15
10
5
0
♀
♂
35/40
35/35
10/10
16/16
6/34
**
22/37
**
2/9
10/14
**
NaCl
Neo
Physo
Pilo
Treatment

Figure 4: Time course of hypothermia induced by 5 mg/kg pilocarpine i.p. in unrestrained normoxic male and female mice. Rectal temperatures were measured intermittently before and after pilocarpine injection. Each value represents the ($\bar{x}$ ± S.E.) of body temperature in 5 male and 5 female mice. Significant differences from pretreatment control temperatures:

$^{**}p < 0.001$.

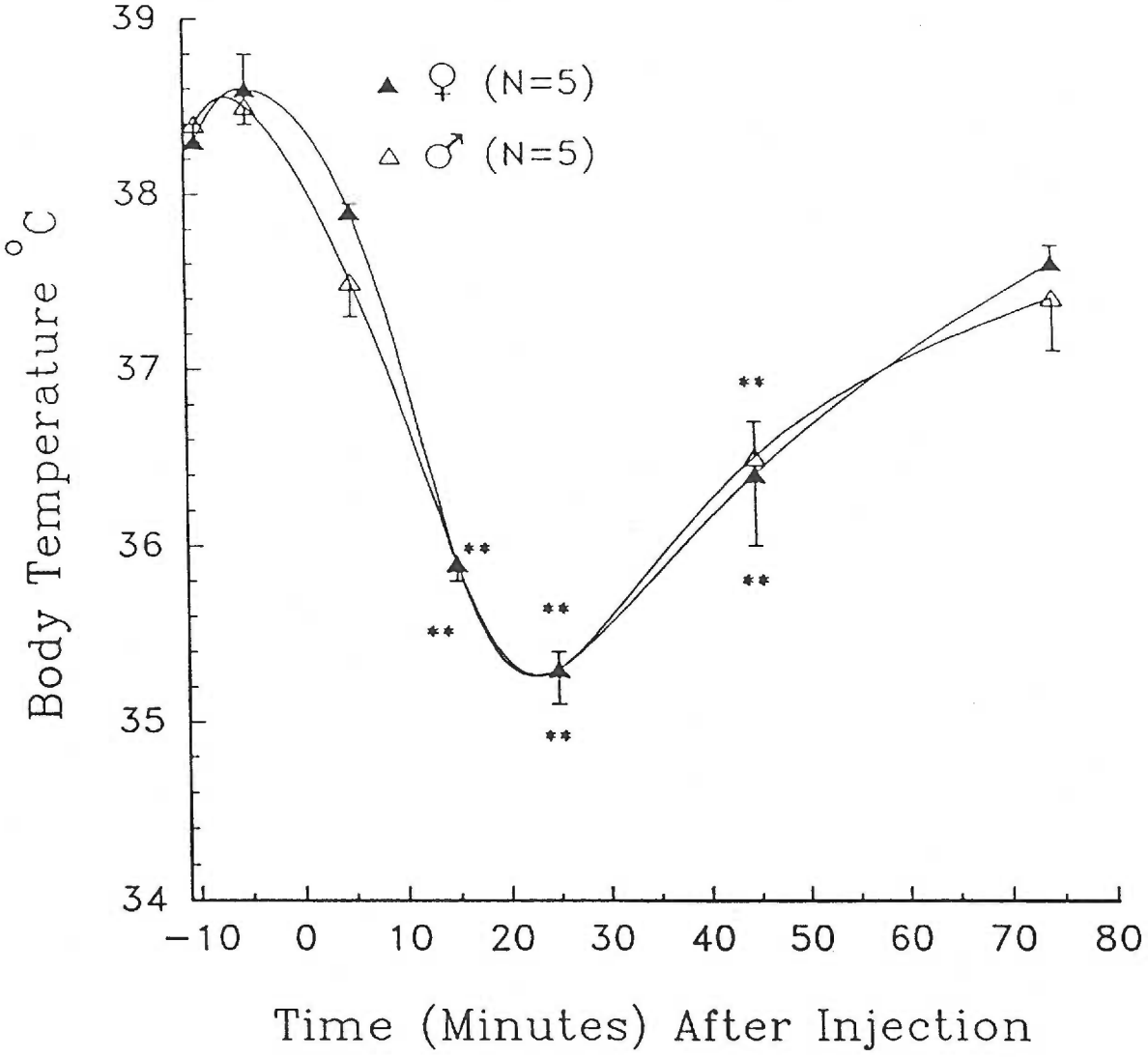

39
38
37
36
35
34
Body Temperature °C
▲ ♀ (N=5)
△ ♂ (N=5)
**
−10
0
10
20
30
40
50
60
70
80
Time (Minutes) After Injection

Figure 5: Time course of hypothermia induced by 0.2 mg/kg neostigmine i.p., in unrestrained normoxic male and female mice. Rectal temperatures were measured intermittently before and after neostigmine injection. Each value represents the ($\bar{x}$ ± S.E.) of body temperature measurement in 4 male and 5 female mice. Significant differences from pretreatment control temperatures were: † $p < 0.05$, †† $p < 0.01$.

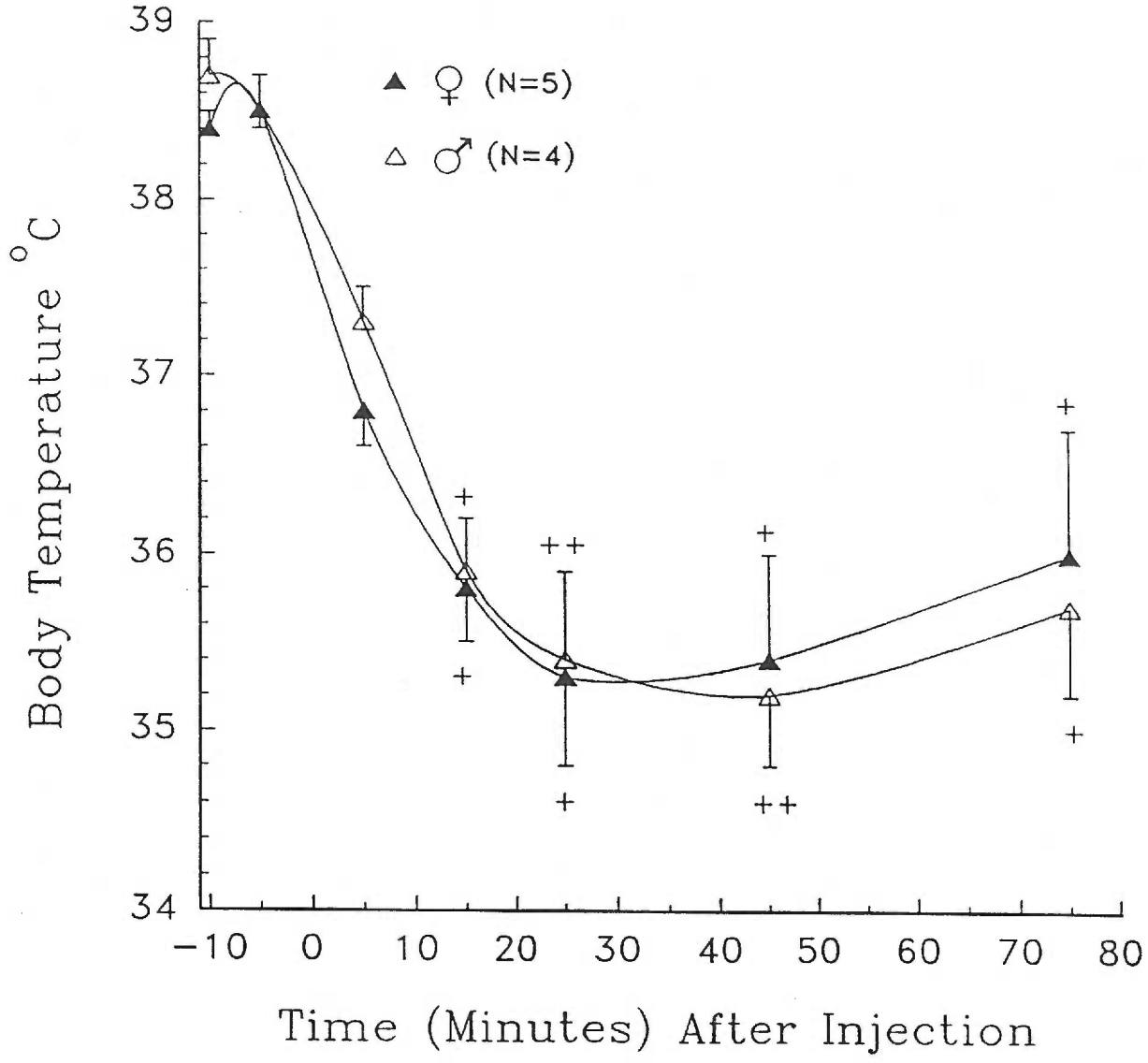
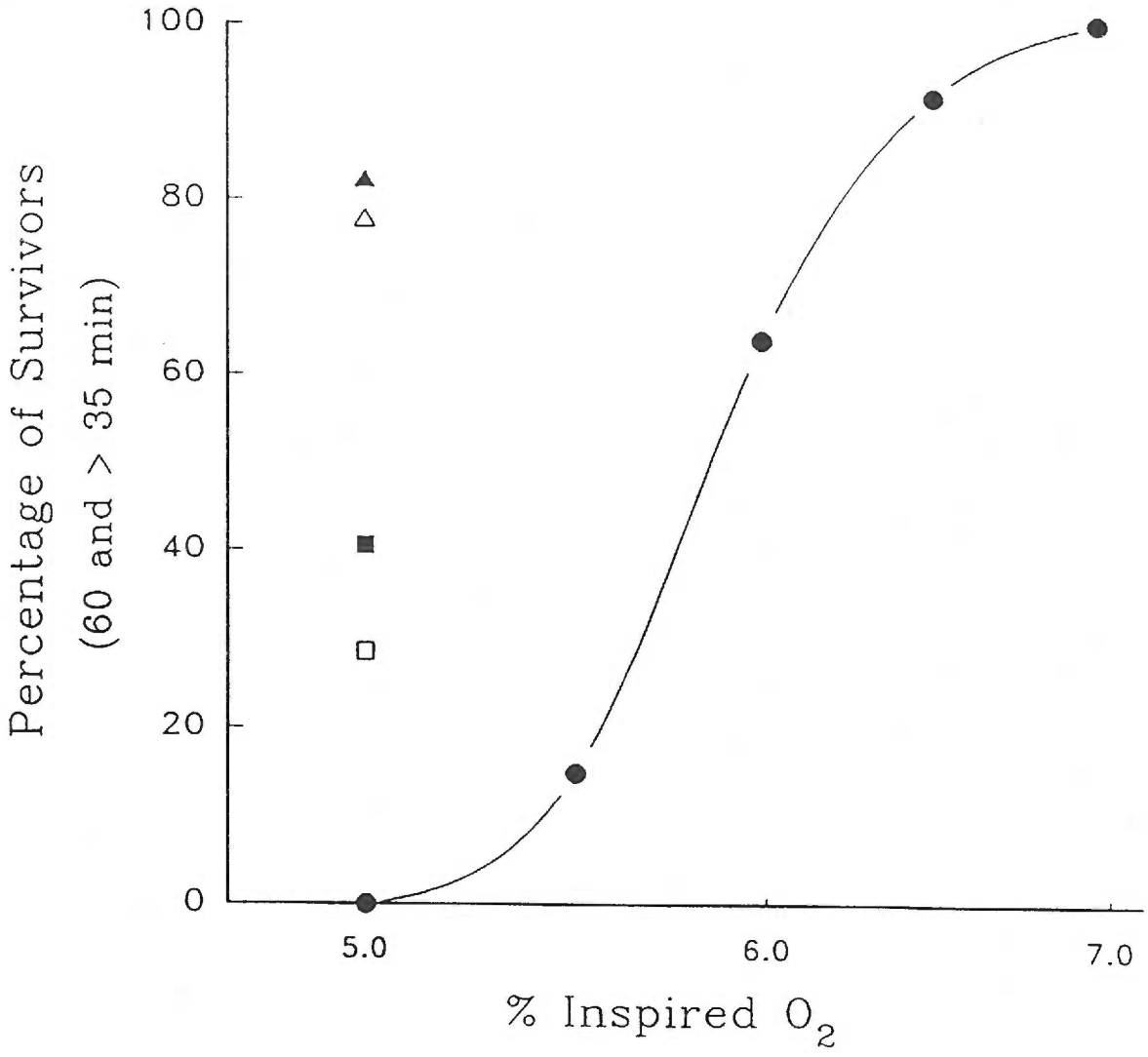

39
38
37
36
35
34
Body Temperature °C
▲ ♀ (N=5)
△ ♂ (N=4)
+
++
+
++
+
+
−10
0
10
20
30
40
50
60
70
80
Time (Minutes) After Injection

Figure 6: The curve describing long term survival (35-60 min) versus inspired O_2 concentration is shifted to the left by physostigmine or pilocarpine treatment. The solid line curve is plotted from the data of Minard and Grant (1982) showing the percentages of NaCl-treated male mice surviving 60 min at each inspired O_2 concentration (log scale). Compare the solid curve with the locations of the 35-60 min survival percentages of physostigmine treated females (▲) and males (■), or pilocarpine treated females (△) and males (□); data from Fig. 2.


Percentage of Survivors
(60 and > 35 min)
100
80
60
40
20
0
5.0
6.0
7.0
% Inspired O_2

INDEX TERMS

Physostigmine, pilocarpine, hypoxia, gender difference

Manuscript III

(To be submitted)

Hypothermia During Hypoxia in Unrestrained, Conscious Mice Treated with Physostigmine: A Gender Comparison

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ABSTRACT

Peritoneally implanted transmitters recorded core temperatures in male and female mice given i.p. physostigmine (0.2 mg/kg) under normoxic and hypoxic (5% O_2 - 95% N_2) conditions. In normoxia females exhibited significantly greater hypothermia than did males starting within 10 minutes after physostigmine. Twenty two animals of each sex were exposed to hypoxia 15 min after physostigmine, and 81.8% of females survived 60 minutes compared to 54.5% of males; a significant difference. During hypoxia core temperatures dropped markedly (7-9°C), with females showing a significantly greater magnitude and rate of hypothermia. Thus, females reached a core temperature of 31°C after 15 minutes of hypoxia, compared to 29 minutes for males. A histogram of hypoxic mortality versus time showed that after 15 min of hypoxia surviving females had a 98% probability of surviving 60 minutes, whereas males had the same survival probability only after 29 minutes of hypoxia. The faster rate of hypoxic hypothermia in females therefore appears to be a major factor in the significant gender difference in percentages of physostigmine treated 60 minutes hypoxia survivors. The discussion considers (1) the hypothesis that a core hypothermia level near 31°C is critical to hypoxic survival in both sexes, and (2) the probable mechanisms in hypothermic protection against hypoxia.

INTRODUCTION

Physostigmine, at doses of 0.1 - 0.3 mg/kg, has been reported to prolong the survival time of male mice exposed to severe hypoxia (5% O_2 - 95% N_2) (Scremin and Scremin, 1979; Artru and Michenfelder, 1980; Minard and Grant, 1982). Recently, we showed (Saiyed and Riker, In Press) that the protective effect of physostigmine in severe hypoxia was significantly greater in female compared to male mice, as measured by the percentages of treated mice surviving 60 minutes of exposure to 5% O_2 - 95% N_2. In that study we also measured, by rectal probe, the hypothermic effect of physostigmine under normoxic conditions and found no significant sex difference.

It has long been known that either cold-induced or drug-induced hypothermia confers a protective effect during hypoxia (Kottke et al., 1948; Phillips et al., 1950; Minard and Grant, 1982) and that hypoxia itself induces hypothermia in rodents (Gellhorn, 1937; Kottke et al., 1948; Phillips et al., 1950; Chevillard, 1966; Bhatia et al., 1969). Furthermore, the protective effect of physostigmine, at least in male mice, can be abolished if ambient temperature is raised to near body temperature (Artru and Michenfelder, 1980). All of these observations have led to the general assumption that hypothermia is a major mechanism in drug protection against hypoxia (Artru and Michenfelder, 1980; Minard and Grant, 1982).

Prior to our previous study (Saiyed and Riker, In Press) there had not been a systematic gender comparison of physostigmine protection against

hypoxia. Equally important, there had previously been no systematic gender comparison of hypothermia induced by cold, drugs, or hypoxia. As part of our search for mechanisms underlying the gender difference in physostigmine protection against hypoxia it was essential that we measure core temperature during hypoxia in physostigmine-treated male and female mice. Because the gender difference in physostigmine prolongation of survival was demonstrated in conscious, unrestrained mice it was also essential that core temperatures during hypoxia be measured in conscious unrestrained mice; and measured without the handling and artifacts incident to rectal probe techniques. Therefore, we have utilized the technological advantage of peritoneally implanted temperature sensitive transmitters to achieve continuous recording of core temperatures in conscious, unrestrained and unhandled mice. The present investigation demonstrates the very large magnitude hypothermia, approximately ten degrees C below control, that occurs during hypoxia in physostigmine treated mice. Additionally, the degree of hypothermia in physostigmine treated female mice is significantly greater than that in males under both normoxic and hypoxic conditions.

METHODS

Animals: Thirty four male and an equal number of female white Swiss Webster mice (Bantin and Kingman, Oxnard, CA) weighing ($\bar{X}$ ± S.E.M.) 36.8 ± 0.4 and 32.5 ± 0.3 grams, respectively, were used in the present study. Food and water were provided ad libitum during a light dark cycle of 14/10 hours. The mice (4 to 5 per shoe box) were housed in the AALAC accredited animal care facility at this institution and were acclimatized at least for one week prior to use in the experiments. The protocol for experiments reported here was reviewed and approved by the institutional Animal Care Committee.

Experimental Hypoxia: A plexiglass 1 liter cylinder (Diameter 9 cm, Length 16.5 cm) with removable lids at either end was used as the hypoxic chamber. One lid had three ports for gas flow inlet and outlet, and for holding a thermometer to measure chamber temperature. The opposite removable lid, without ports, was used for introduction of experimental mice. The humidified gas mixture (5% O_2 - 95% N_2) (Airco Medical Gases, Murray Hills, N.J.) flowed at a rate of 2 L/min, starting 5 minutes before introduction of mice, and then continuously throughout the duration of survival. The maximum time of exposure to hypoxia was limited to 60 minutes. Survival time was measured to the nearest 0.1 minute by digital stop watch, from the time of introduction into the chamber until complete cessation of respiration. All experiments were conducted between 0800 and 1700 at ambient room temperature, 24.0 ± 0.1°C ($\bar{X}$ ± S.E.M.). A

regression analysis showed no relationship between the slight day to day variations in ambient or chamber temperature and survival time.

Treatment Protocol: Mice were injected (i.p.) either with NaCl 0.9% or physostigmine hemisulfate 0.2 mg/kg (Sigma Chemical Co., St. Louis, MO) and injection volumes were kept within the range from 4 to 8 μl/g. For the experiments involving exposure to hypoxia, injections of NaCl or physostigmine were made 15 min prior to introduction of mice into the hypoxia chamber.

Body Temperature Measurement: Body temperature was monitored continuously in normoxia and during hypoxia by temperature sensitive miniature transmitters, coated with Paraffin/Elvax (Minimitter Co., Sunriver, OR), implanted in the peritoneal cavity. The transmitters are simple resistor-capacitor oscillators in which the resistor value varies with temperature. Thus, the output frequency of the oscillator is temperature dependent. Since each transmitter has a unique frequency output as a function of temperature, a calibration curve was carefully determined for each transmitter before implanting in the peritoneal cavity. Calibration was carried out in an insulated water bath (225 ml), the temperature of which was altered in 2-3°C increments over the range from 25 to 40°C. After 5 minutes of equilibration at each new temperature setting the frequency output was recorded from the digital display of a frequency counter (Heath Model 2372) connected to the receiver (Model RA 1010, Minimitter Co., Sunriver, OR)

placed underneath the water bath. The relationship between the temperature and *ln* frequency was determined for each transmitter by regression analysis to obtain the Y intercept and the slope. For recording from the implanted calibrated transmitters the receiver was placed beneath the hypoxia chamber and the temperature of the mice was calculated from the recorded frequency by the following formula: Temperature °C = (*ln* Frequency - Constant) / Slope.

In a few animals with transmitter implants rectal temperatures were also measured intermittently by a petrolatum-lubricated thermocouple (ret-3) inserted into the rectum to a depth of ¾ inch. The rectal temperatures were read from the digital display monitor (Thermalert, Model TH-8, Sensortek, Clifton, NJ) connected to the thermocouple probe.

Implantation of Transmitters: Mice were anesthetized by methoxyflurane, abdominal hairs were shaved, and the abdomen was opened by a 1-1.5 cm midline incision. The calibrated, paraffin-coated transmitters (weight, approximately 2 g) were placed in the peritoneal cavity, and secured by ligature to the anterior abdominal muscles. The abdomen was closed by suturing the abdominal muscles and skin separately, followed by application of Betadine® to the wound. The whole procedure for transmitter implantation (from the start of anesthesia to closure of the wound) was completed in 10 to 15 minutes. Animals with transmitter implants were allowed a post-operative recovery period of at least 15 days before they were used for experiments. This minimum period

of post-operative recovery was based on analysis of daily body weights in 10 male and 10 female mice that had received transmitter implants. In these 20 animals peak weight loss (approximately ten percent) occurred on the third post-operative day, after which they returned to a normal weight gain mode of slightly greater than one percent of body weight per day. Thus, they regained their pre-operative body weights by the eighth post-operative day.

Data Analysis: The body temperature measurements (°C) were calculated and expressed as mean ± S.E.M. The hypoxic survival data is presented as mean ± S.E.M. survival time in minutes, and also as the percentage of animals surviving the specified duration of hypoxia. The difference between the means of two experimental groups was evaluated for significance by analysis of variance. For the data on the percentage of animals surviving 60 minutes of hypoxia the differences between the groups were evaluated by chi-square and Fisher's exact test (Dawson-Saunders and Trapp, 1990). The minimum requirement for statistical significance was taken as $p < 0.05$.

RESULTS

Comparison of Physostigmine Hypothermia by Core and Rectal Temperature Measurements in Normoxic Mice.

Three male and three female mice with peritoneal transmitter implants were injected (i.p.) with physostigmine 0.2 mg/kg under conditions of room air and room temperature (24.9 ± 0.2°C). In addition to continuous recordings of the transmitter signals pre and post drug administration, each mouse had rectal temperature measurements made before and at 5, 15, 25, 45 and 75 minutes after physostigmine. Figure 1 shows the time course of hypothermia measured by the two methods. The time course shown in figure 1 was virtually identical with that reported by us previously (Saiyed and Riker, In Press) using only rectal temperature measurements. Therefore, there was no apparent alteration in physostigmine pharmacokinetics as a consequence of the implant surgery and post-operative recovery.

Figure 1 also confirms our previous observations (Saiyed and Riker, In Press) that female body temperature is consistently lower than that in males. The sample size in figure 1 is too small for meaningful statistical evaluation, but a subsequent section (Core Temperature During Hypoxia) of this report, involving larger numbers of animals, will demonstrate that the gender difference is statistically significant during both normoxia and hypoxia. A second noteworthy point in figure 1 concerns the relative differences between core and rectal temperatures in males and females. In males the mean (± S.E.M.) core-

rectal temperature difference for the 6 measurement time points was -0.88 ± 0.12, compared to -0.27 ± 0.16 for females; a statistically significant ($p < 0.005$) gender difference.

Continuous Measurement of Core Temperature During Hypoxia in Male and Female Mice.

Physostigmine 0.2 mg/kg was administered, i.p., to 22 female and 22 male mice with calibrated, functioning transmitter implants, 15 min before placement in a hypoxic (5% O_2 - 95% N_2) atmosphere. Eighteen of the 22 females (81.8%) and 12 of the 22 males (54.5%) survived one hour of hypoxia. The 4 females that did not survive through one hour had a survival time ($\bar{X}$ ± S.E.M.) of 14 ± 3.3 min, compared to 16.8 ± 2.0 min for the 10 short-survival males.

Figure 2 is a plot of the recorded core temperatures in a control period (10 min before physostigmine), followed by a 15 min period after physostigmine, 0.2 mg/kg, administration, and then throughout the 60 min period of hypoxia. The data for the time periods prior to the start of hypoxia is derived from 25 transmitter-implanted animals of each sex; the 22 subjected to hypoxia plus the 3 used for the normoxia experiment in figure 1.

As shown in figure 2, even within 5 minutes after physostigmine there was a highly significant difference ($p < 0.001$) between male and female core temperatures. This significant difference increased, so that at the start of

hypoxia, 15 minutes after physostigmine, the core temperature of 35.55 ± 0.15 ($\bar{X}$ ± S.E.M.) in females was 0.73°C lower than that in males (36.28 ± 0.19). During hypoxia there was a progressive, intense hypothermia in which the significant gender difference was not only maintained but expanded. Thus, after 60 minutes of hypoxia the females had a core temperature 1.92°C lower than that of the males (Fig. 2). Presumably this ultimate separation of 1.92°C is attributable to the steeper slope of the hypothermic response to hypoxia in females.

The significantly steeper slope of the hypothermic response to hypoxia in physostigmine-treated females is clearly illustrated in figure 3. In this figure the decreases in core temperature, relative to time 0 of hypoxia, are plotted against the log of time. It can be seen that at 5 minutes after the onset of hypoxia the slope of the hypothermic response in females begins to increase sharply compared to the slope in males (Fig. 3). Regression analysis of the data between 5 and 60 minutes yielded slope values of 5.496 and 4.422 for females and males, respectively. This slope difference was highly significant ($p < 0.005$).

We were also interested in determining whether the approximately linear rate of decrease in body temperature during the early stage of hypoxia (Figs. 2, 3) was influenced by the physostigmine pretreatment. Therefore, a separate group of mice with implanted transmitters (7 males and 5 females) were injected with 0.9% NaCl 15 min before hypoxia exposure. Figure 4 shows that

there was no significant difference between physostigmine treated and NaCl treated mice of either sex in the rate of change in body temperature, at least within the first 5 minutes of hypoxia. The 5 minute time period for comparison of NaCl- and physostigmine-treated mice is imposed by the well known fact that NaCl-treated mice survive only a few minutes in an atmosphere of 5% O_2 - 95% N_2 (Scremin and Scremin, 1979; Minard and Grant, 1982; Saiyed and Riker, In Press). In the present case (Fig. 4) the NaCl-treated 7 males and 5 females had survival times, respectively, of 4.6 ± 0.6 and 8.3 ± 2.0 min ($\bar{X}$ ± S.E.M.); not significantly different.

Gender Difference in One Hour Hypoxia Survivors.

In a previous publication (Saiyed and Riker, In Press) concerning the significant gender difference in physostigmine prolongation of survival (female > male) during hypoxia we reported the extremely high probability that a treated animal surviving 35 minutes of hypoxia would survive 60 minutes (see also Scremin and Scremin, 1980). The present experiments validated the high probability since the physostigmine treated mice that survived as long as 35 minutes did survive to 60 minutes. We chose the 60 minute exposure time for the present experiments, however, to obtain the maximum information about gender-related hypothermia during hypoxia.

Figure 5 presents the cumulative data from our present and previous (Saiyed and Riker, In Press) studies of 60 minute hypoxia survival, showing the

highly significant ($p < 0.00001$) gender difference between the percentage (83.8) of physostigmine-treated females and the percentage (39.0) of physostigmine-treated males that survived hypoxia for one hour. In addition, figure 5 presents the cumulative data from this large series in the form of a mortality histogram with respect to length of exposure to hypoxia. The mortality-time histogram reveals still another aspect of gender difference; namely, that the females that survived through the first 15 minutes have a 98% chance (66/67) of surviving for one hour. In contrast, male mortality continues through the first 30 minutes of hypoxia, after which the surviving males also have a 98% chance (40/41) of surviving one hour. This difference between female and male mortality as a function of time during hypoxia has a striking correlation with the difference between female and male hypothermia (Figs. 2 and 3). The relationship between these two phenomena will be considered and amplified in the Discussion section.

DISCUSSION

In 1979 Scremin and Scremin reported that physostigmine, compared to saline, pretreatment significantly prolonged the survival times of male mice exposed to severe hypoxia (5% O_2 - 95% N_2), and a fraction of the physostigmine treated mice survived a full 60 minutes of hypoxia. Shortly thereafter, Artru and Michenfelder (1980) showed that the protective effect of physostigmine against hypoxia in male mice was abolished if the experiments were conducted at ambient temperatures (35°C) near body temperature. The magnitudes of hypothermic responses in rodents to drugs such as physostigmine (Minard and Grant, 1992) and to stresses such as hypoxia (Gellhorn, 1937; Kottke et al., 1948) are known to vary inversely with ambient temperature (Chevillard, 1966). Since the experiments of Scremin and Scremin (1979) were conducted at usual room temperature Artru and Michenfelder (1980) concluded that the prolonged survival during hypoxia was attributable to hypothermia from both physostigmine and hypoxia. However, neither the study of Scremin and Scremin (1979), nor that of Artru and Michenfelder (1980) included measurements of body temperature.

We have previously reported (Saiyed and Riker, In Press) a significant gender difference in prolonged hypoxic survival of mice after physostigmine (0.2 mg/kg) treatment, and the present paper summarizes (Fig. 5) our total experience to date wherein 83.8% of females compared to 39.0% of males survived 60 minutes of exposure to 5% O_2 - 95% N_2. Prior to the present study

we had made rectal temperature measurements only in physostigmine-treated normoxic mice, demonstrating significant hypothermia, but no significant gender difference (Saiyed and Riker, In Press). Therefore, the significant gender difference in survival was apparently not related to a gender difference in physostigmine-induced hypothermia. That interpretation, however, was limited by two important considerations. First, the question remained whether males and females differed in the rate and/or degree of hypothermia during hypoxia. Second, the handling, stress and even minimal restraint associated with rectal temperature measurement is well known to influence body temperature (Briese and deQuijada, 1970; Martin et al., 1977; Clark and Clark, 1980; Cunningham and Bischof, 1987), thereby introducing relatively large variation and possibly obscuring a gender difference in physostigmine hypothermia. The present study was undertaken to solve both these problems. Thus, we believe this is the first report of nonintrusive continuous core temperature measurement during hypoxia in unrestrained, conscious, unhandled mice; a report that also includes the first systematic gender comparison for the hypothermic effects of physostigmine and of hypoxia. The results demonstrate that female mice have significantly greater hypothermia than males not only after physostigmine, but also during hypoxia. Although hypothermia may not be the sole mechanism underlying prolonged survival of mice exposed to severe hypoxia, the present results suggest that it is the critical factor in the gender difference in survival of physostigmine-treated mice.

For example, figure 5 demonstrates that the first 15 minutes of hypoxia is a critical time interval for females, so that those who survive the first 15 minutes are virtually certain to survive a full hour of hypoxia. At the 15 minute time point females have a mean core temperature of 31.2°C (Fig. 2), practically seven degrees below the control core temperature. In females, therefore, 31°C appears to be the critical absolute level of hypothermia required for long survival during hypoxia. If we assume that 31°C is also a critical absolute level in males, figure 2 reveals that males, consistent with their slower rate of hypothermia (Fig. 3), attain the 31°C temperature only after 28 minutes of hypoxia. Correspondingly, figure 5 demonstrates that males that survive the first 29.9 minutes of hypoxia are also virtually certain to survive 60 minutes. The striking correlation, in males and females, between the times at which hypoxic mortality ends and the core temperatures at those times supports the assumption that there is a common critical level of hypothermia in both sexes. Consequently, we hypothesize that the gender difference in the percentages of one hour survivors of hypoxia rests predominantly on the significantly slower rate of development of hypoxic hypothermia in males.

The preceding hypothesis could be tested by nullifying, or compensating for, the gender difference in rate and degree of hypothermia after physostigmine and hypoxia, with the aim of achieving equal temperatures in males and females at an equal time. One approach would be to select time intervals between physostigmine administration and onset of hypoxia that are

different for males and females. From the data in figures 1-3 it can be predicted that with a physostigmine-to-hypoxia interval of 20 minutes for males and 7-8 minutes for females both sexes would achieve a temperature near 31°C 15 minutes into hypoxia. Another approach would be to conduct the experiments at different, but constant, ambient temperatures for males and females since the degree and rate of hypothermia in mice depends on ambient temperature (Chevillard, 1966). A third option would utilize another pharmacological gender difference. Atropine methylnitrate pretreatment does not affect physostigmine hypothermia in male mice, but attenuates the hypothermia in females (Saiyed and Riker, unpublished observation). Therefore, pretreatment of females with an appropriate dose of atropine methylnitrate, prior to physostigmine, could likely equalize their hypothermic responses to that of males.

In summary we have measured core temperatures in unrestrained, unhandled, conscious male and female mice treated with physostigmine and subsequently exposed to severe hypoxia (5% O_2 - 95% N_2). The results show that physostigmine itself produces significantly greater hypothermia in females, and that this gender difference is significantly expanded during hypoxia. The results further suggest that attainment of a low core temperature, near 31°C, is critical to both sexes for long term survival in severe hypoxia. The importance of hypothermia in protection against hypoxia rests primarily on its ability to reduce oxygen consumption, especially in brain (Carlsson et al., 1976; Hägerdal et al., 1975), and on its ability to shift the oxyhemoglobin dissociation curve to the left

(Brown and Hill, 1923; Turek et al., 1978). Both of these effects of hypothermia result in increased efficiency of oxygen utilization when supply is limited. Assuming that the mouse, like the rat (Hägerdal et al., 1975) has a Q_{10} of 2 for cerebral oxygen consumption (5% decrease per 1°C decrease) a body/brain temperature drop from 38 to 31°C would reduce cerebral oxygen, as well as glucose (Michenfelder and Theye, 1968), consumption by 35%. The fact that the present study indicates that females have a much faster drop in temperature, hence in cerebral oxygen consumption, than do males strongly suggests that future research in the fields of hypoxia and ischemia should focus considerably more effort on gender comparison than has been done to the present.

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Figure 1. Mean core (circle) and rectal (triangles) temperatures as a function of time after i.p. physostigmine (0.2 mg/kg) in normoxic male (N=3) and female mice (N=3) with peritoneally implanted temperature sensitive transmitters. Error bars at the times of rectal temperature measurement have been omitted for clarity in demonstrating the gender aspect of the core-rectal temperature differences.

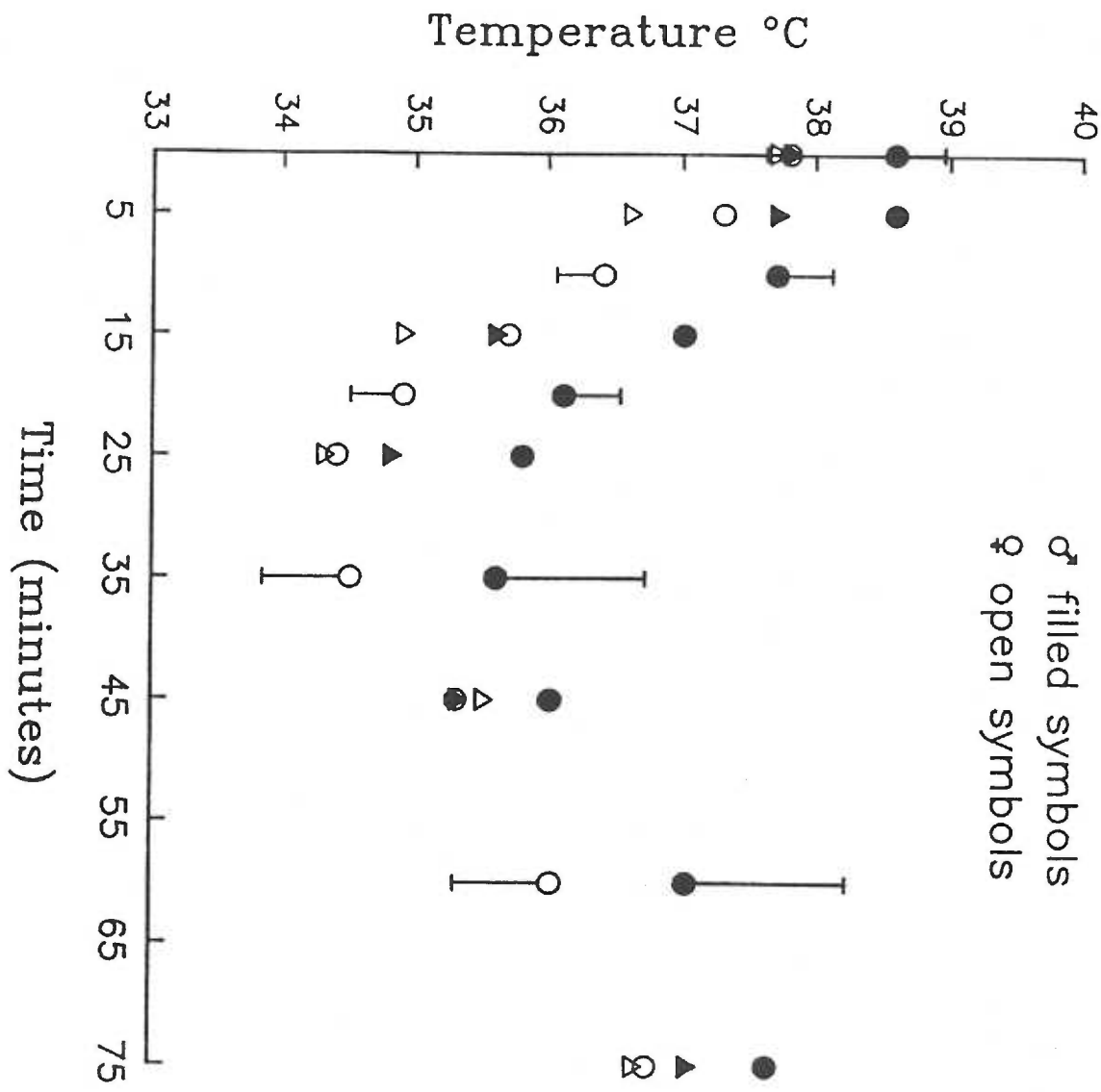

Temperature °C
33
34
35
36
37
38
39
40
Time (minutes)
5
15
25
35
45
55
65
75
♂ filled symbols
♀ open symbols

Figure 2. Core temperatures ($\bar{X}$ ± S.E.M.) before and during 60 minutes of hypoxia (5% O_2 - 95% N_2) in transmitter-implanted male and female mice treated with i.p. physostigmine (0.2 mg/kg) at -15 minutes (↑). Between -25 and 0 minutes N=25 for each sex. Between 0 and 60 minutes (hypoxia) the N value decreases because of intercurrent mortality (see RESULTS section). For females N decreases from 22 to 18, and for males N decreases from 22 to 10. Between -10 and +10 minutes there is a significant (*) gender difference in core temperature, with p varying between < 0.01 and < 0.005. From +10 through 60 minutes the gender difference has p values that range between < 0.005 and < 0.0001.

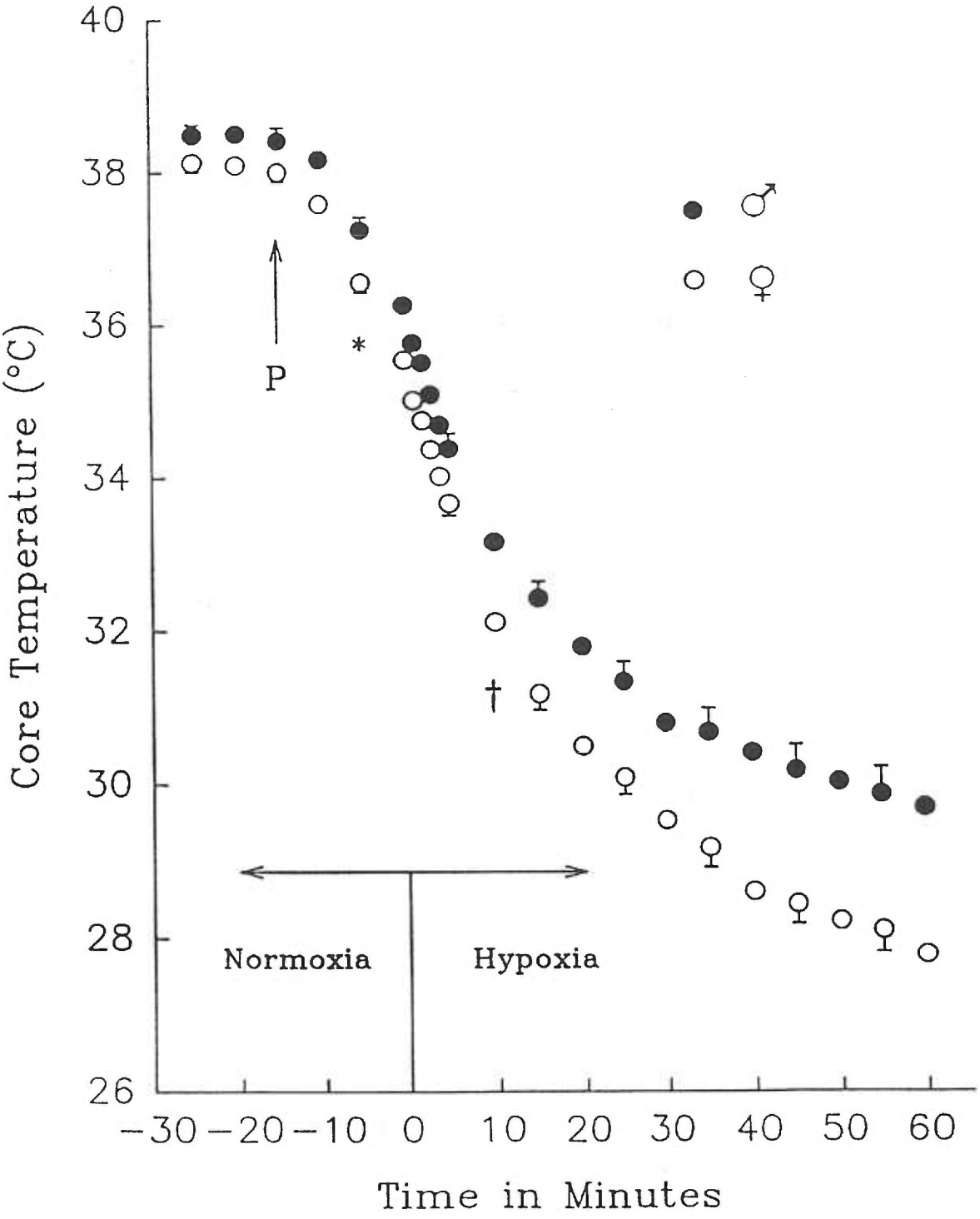

40
38
36
34
32
30
28
26
Core Temperature (°C)
P
*
†
Normoxia
Hypoxia
♂
♀
−30 −20 −10 0 10 20 30 40 50 60
Time in Minutes

Figure 3. Regression analysis of the decrease in core temperature during 5 to 60 minutes of hypoxia (5% O_2 - 95% N_2) in physostigmine-treated male and female mice. Ordinate: decrease in temperature, in °C, relative to core temperature at time 0 (onset of hypoxia). Abscissa: log scale of time. The figure is a plot based on the same data shown in figure 2, to illustrate the significantly ($p < 0.005$) steeper hypothermia slope (5.496) in females compared to males (4.442) between 5 and 60 minutes.

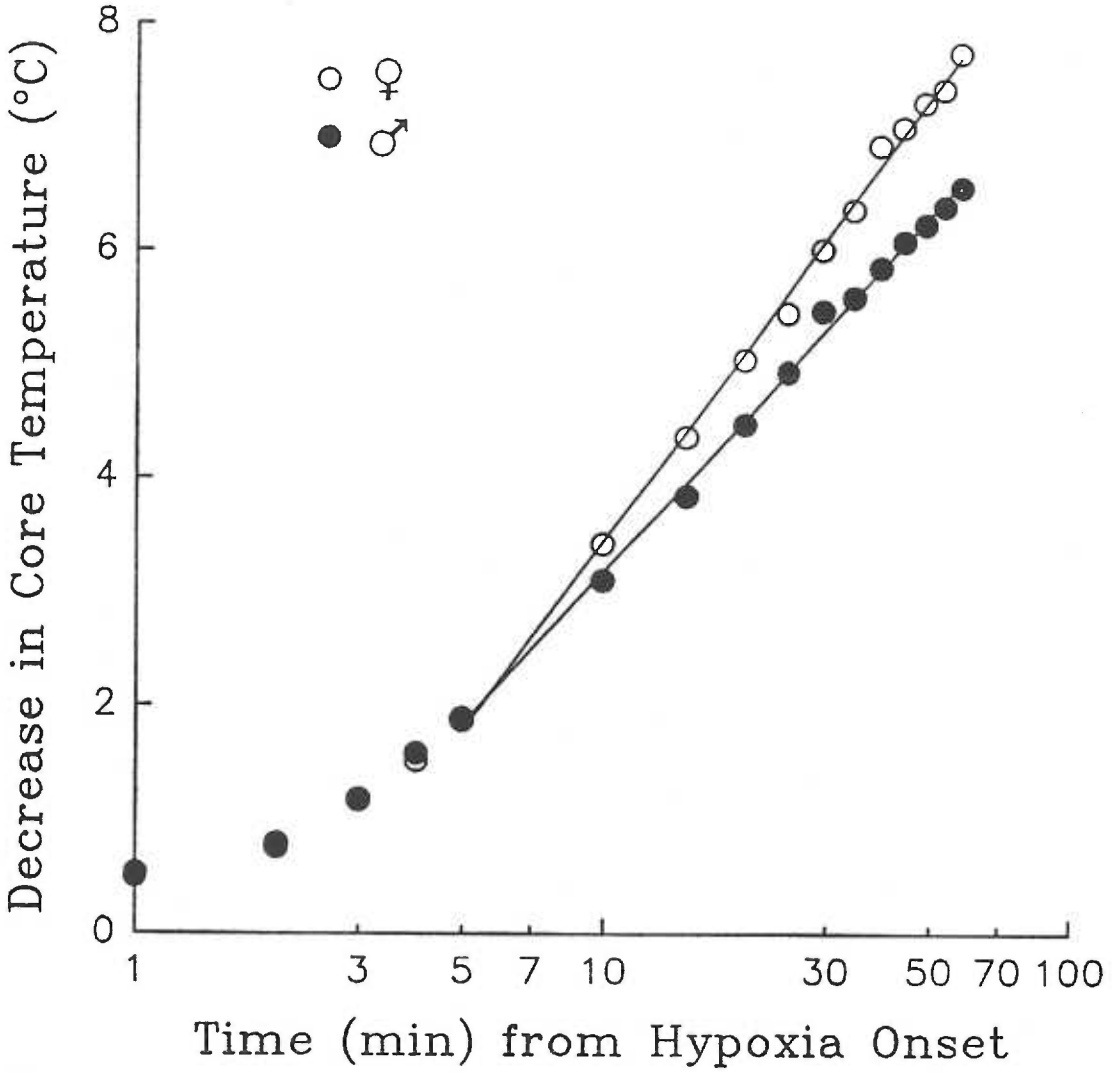

Decrease in Core Temperature (°C)
0
2
4
6
8
♀
♂
1
3
5
7
10
30
50
70
100
Time (min) from Hypoxia Onset

Figure 4. Comparison of the rates of hypothermic responses during the first 5 minutes of hypoxia (5% O_2 - 95% N_2) in saline (S) treated (5 females, 7 males) and physostigmine (P) treated male and female mice with implanted peritoneal temperature transmitters. Data for physostigmine treated mice is that shown in figure 2. Slope values obtained from linear regression varied between -0.4 and -0.5, and were not significantly different.

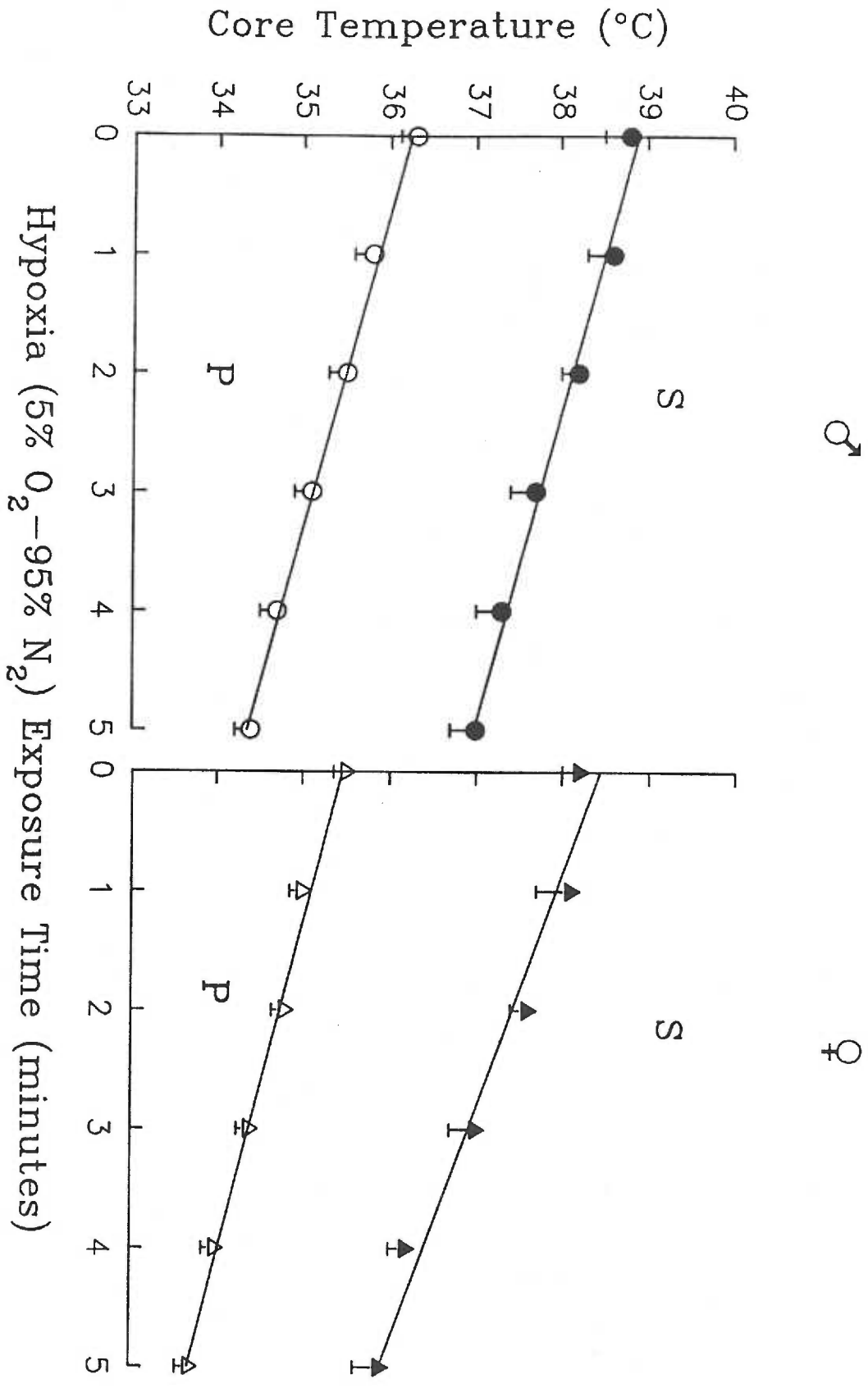
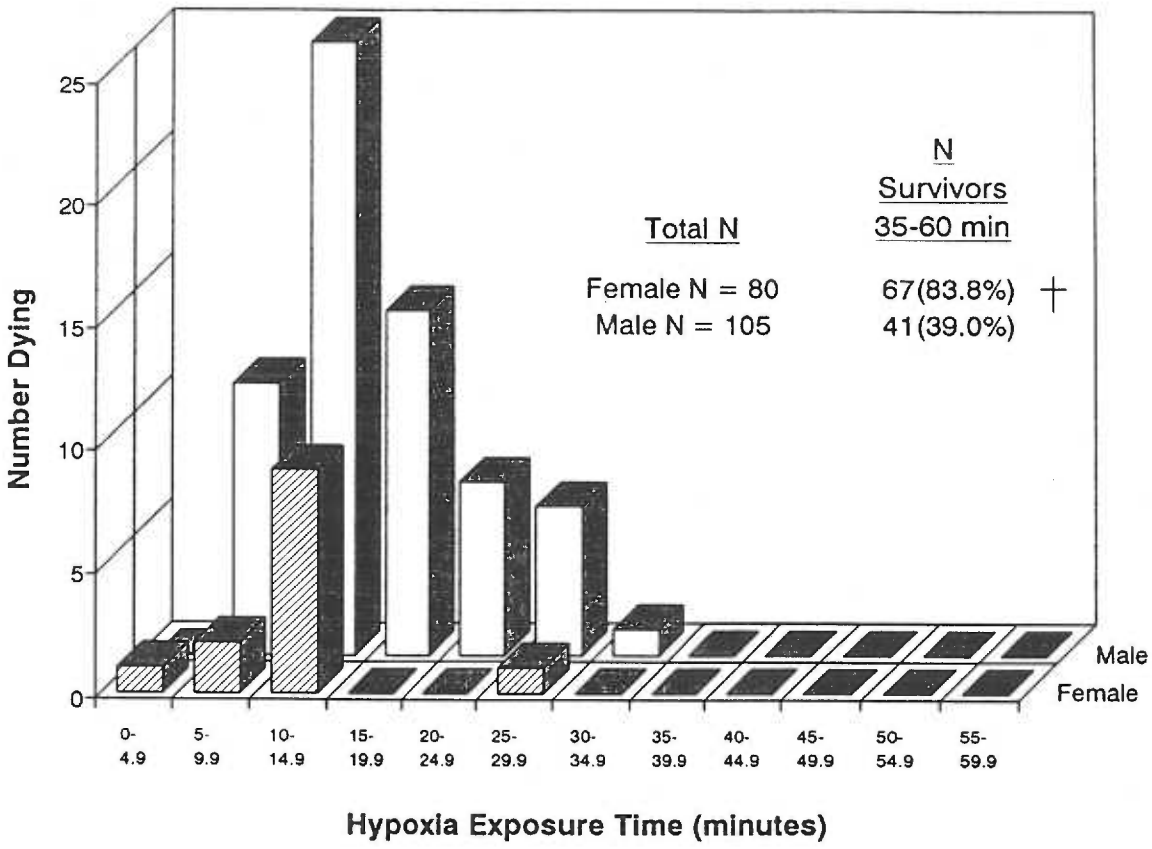

Core Temperature (°C)
33
34
35
36
37
38
39
40
0
1
2
3
4
5
P
S
♂
♀
Hypoxia (5% O_2–95% N_2) Exposure Time (minutes)

Figure 5. Histogram of mortality versus time in physostigmine treated male and female mice exposed to severe hypoxia (5% O_2 - 95% N_2). The total number of animals represent the data from the present and previous (Saiyed and Riker, In Press) studies. Note the highly significant ($p < 0.00001$) gender difference (†) in the percentages of 1 hour survivors. Also note that female mortality occurs predominantly within the first 15 minutes compared to 30 minutes for males.


Number Dying
25
20
15
10
5
0
N
Survivors
Total N
35-60 min
Female N = 80
67(83.8%)
Male N = 105
41(39.0%)
Male
Female
0-
4.9
5-
9.9
10-
14.9
15-
19.9
20-
24.9
25-
29.9
30-
34.9
35-
39.9
40-
44.9
45-
49.9
50-
54.9
55-
59.9
Hypoxia Exposure Time (minutes)

Manuscript IV

(To be submitted)

The Effects of α and β Adrenergic Blockers on Physostigmine Protection Against Hypoxia

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ABSTRACT

The purpose of the present study was to examine the role of α and β adrenergic activation by physostigmine in its protective effect against hypoxia in male and female mice. Survival times were measured in groups of control and treated (i.p. injection) animals exposed to hypoxia (5% O_2 - 95% N_2). Body temperatures during normoxia in control and treated animals were measured by rectal probe. In the animals exposed to hypoxia the body temperature was monitored by temperature sensitive transmitters implanted in the peritoneal cavity. The survival times of the saline-treated control animals were 4.5 ± 0.3 (N=42) minutes for males, and 5.4 ± 0.3 (N=43) minutes for females. Physostigmine (0.2 mg/kg, i.p.) prolonged the survival time of both male and female mice, but a significantly greater proportion of physostigmine treated females (91.7%) than males (32.4%) survived through 35 minutes of hypoxia. This protective effect of physostigmine was completely abolished by pretreatment with the α adrenergic blocking drugs, phentolamine and prazosin (4 mg/kg, i.p.) in both male and female mice. In contrast, the β adrenergic blocking drug propranolol (10 mg/kg, i.p.) did not alter the protective effect of physostigmine in either sex. The significant hypothermic action of physostigmine, considered to be an important factor in survival during hypoxia, was unaltered by both α and β adrenergic blocking drugs. The results are discussed in relation to the sympathoadrenal activation produced by physostigmine, and it is concluded that the maintenance of blood pressure through intact α adrenergic pathways is critical for survival during hypoxia.

INTRODUCTION

Physostigmine is known to protect mice against lethal hypoxia (5% O_2 - 95% N_2) (Scremin and Scremin, 1979; Artru and Michenfelder, 1980; Scremin and Scremin, 1980; Minard and Grant, 1982; Riker et al., 1990; Saiyed and Riker, In Press). This protective effect is primarily mediated by physostigmine activation of central cholinergic pathways. Therefore, physostigmine protection can be blocked by anticholinergic drugs that cross the blood brain barrier, for example atropine sulphate (Scremin and Scremin, 1980; Riker et al., 1990; Saiyed and Riker, In Press).

We have reported previously that the extent of physostigmine protection against hypoxia is also gender dependent, being significantly greater in female compared to male mice (Riker et al., 1990; Saiyed and Riker, In Press). Although central cholinergic activation by physostigmine is one prerequisite for protection and for the gender difference in survival the precise mechanisms involved remain undefined. Physostigmine can decrease the cerebral metabolic rate of oxygen consumption (Scremin, et al., 1978; Scremin, et. al, 1983), increase cerebral blood flow (Scremin, et al., 1978; Hoffman, et al., 1986) and reduce body temperature (Artru and Michenfelder, 1980; Minard and Grant, 1982; Saiyed and Riker, In Press). All of these effects have been proposed as potential mechanisms of the drug's protection against hypoxia.

However, it is also known that physostigmine's central actions evoke a widespread peripheral sympathoadrenal discharge (Varagić and Kristić, 1966).

Nevertheless, this aspect of physostigmine's actions has not been examined previously for its possible role in protection against hypoxia. The present study was therefore designed to examine the role of physostigmine induced adrenergic activation in protection against hypoxia. We have investigated, in male and female mice, the effects of alpha (phentolamine and prazosin) and beta (propranolol) adrenergic blocking drugs on both the antihypoxic and hypothermic actions of physostigmine. We report here that the hypothermic effect of physostigmine was not altered by either alpha or beta adrenergic blockers. However, physostigmine protection against hypoxia was completely abolished by alpha blockers but was not altered by beta blockers. The results demonstrate that physostigmine-induced activation of peripheral alpha adrenergic pathways is an essential part of its protective action in hypoxia.

METHODS

Animals. Male and female white Swiss Webster mice (Bantin and Kingman, Fremont, CA), weighing 31.2 ± 0.2 g ($\bar{x}$ ± S.E.M.) were used in the present study. Food and water were provided ad libitum during a light/dark cycle of 14/10 hours. The mice (4 to 5 per shoe box) were housed in the AALAC-accredited animal care facility at this institution and were acclimatized for 1 week prior to use in experiments. The protocol for experiments reported here was reviewed and approved by the institutional Animal Care Committee.

Experimental hypoxia. A plexiglass cylinder (length 15 cm, diameter 9 cm), with air tight removable lids was used as the hypoxia chamber. One of the cylinder lids had three ports; one each for gas flow inlet and outlet and the third for holding a thermometer to measure the chamber temperature. The removable lid at the opposite side of the cylinder was used for introduction of experimental mice.

The hypoxic gas mixture (5% O_2 - 95% N_2) (Airco Medical Gases, Murray Hills, N.J.) was humidified by bubbling through water prior to its entry into the hypoxia chamber. The gas flow rate was 2 L/min, starting 5 minutes before introduction of each mouse, and then continuing throughout the duration of survival. The maximum duration of hypoxic exposure was limited to either 35 or 60 minutes. Survival time of mice was measured to the nearest 0.1 minute by a

digital stopwatch, from the time of introduction into the chamber until complete cessation of respiration. All experiments were conducted between 0800 and 1700 at ambient room temperature, 23.3 ± 0.1°C ($\bar{x}$ ± S.E.M.).

Treatment protocol. For measuring the effects of treatment on hypoxic survival time, mice were injected (i.p.) either with NaCl 0.9% or physostigmine hemisulphate (0.2 mg/kg) 15 minutes prior to exposure to hypoxia. Studies of adrenergic antagonism were made by injecting (i.p.) mice with saline, propranolol hydrochloride (10 mg/kg), phentolamine mesylate (4 mg/kg) (ICN Biochemicals Cleveland, OH) or prazosin hydrochloride (4 mg/kg) 15 minutes prior to physostigmine injection. In these animals exposure to hypoxia was, as previously, instituted 15 min after the physostigmine injection. All drugs (except prazosin) were dissolved in 0.9% NaCl. For prazosin the stock solution of 25 mg/ml was made in methanol, and was then diluted to 1 mg/ml with NaCl 0.9% for injection. All drugs were injected intraperitoneally, with the injection volume constant at 4 μl/g, and the doses described in the text refer to the salt, not the base. All drugs, except phentolamine, were purchased from Sigma Chemical Co., St. Louis, MO.

Body temperature. Two different methods were employed for body temperature measurements. For some animals body temperature was sensed by a petrolatum-lubricated thermocouple (ret-3) inserted into the rectum to a depth

of ¾ inch in unrestrained animals, and read from the digital display monitor (Thermalert model TH-8, Sensortek, Clifton, NJ) coupled to the probe. In these cases the rectal temperature was measured intermittently , before and at intervals up to 75 minutes after physostigmine injection. The second method provided continuous core temperature measurement obtained from temperature sensitive transmitters (Minimitter Co, Sunriver, OR) surgically implanted in the peritoneal cavity. The implantations were made at least 15 days prior to experiments. Details of the operative procedure for minimitter implantation have been described previously (see the preceding manuscript III in this thesis).

Data analysis. The body temperature measurements, in °C, were calculated and expressed as the $\bar{x} \pm$ S.E.M. The hypoxic survival data is presented as $\bar{x} \pm$ S.E.M. survival time in minutes, or as the percentage of animals surviving 35 minutes of hypoxia. The differences between the means of two experimental groups were evaluated for significance by t-test. However when more than two groups were involved in the comparison the significance of differences was evaluated by analysis of variance (ANOVA) with subsequent Bonferroni t-test (Dawson-Saunders and Trapp, 1990). For the data on the percentages of animals surviving 35 or 60 minutes of hypoxia the differences between groups were evaluated by chi-square and Fisher's exact test. In all cases a p value < 0.05 was considered the minimum for statistical significance.

RESULTS

I.Survival Time During Hypoxia:

1.Control and Physostigmine Treated Mice:

The mean (± S.E.M.) survival time of the control animals injected with NaCl 0.9% 15 minutes prior to hypoxia was 4.5 ± 0.3 (N=42) minutes for male and 5.4 ± 0.3 (N=43) minutes for female mice. There was no statistically significant gender difference in the control survival times, and none of the control male or female mice survived 35 minutes of hypoxia. After 0.2 mg/kg physostigmine, however, 91.7% (11/12) of females compared to 32.4% (11/34) of males survived 35 minutes of hypoxia (Fig. 1). This gender difference was highly significant ($p < 0.001$). The survival time of the 23 male mice that did not survive 35 minutes of hypoxia was 17.0 ± 1.4 minutes (Fig. 2), significantly ($p < 0.001$) greater than the survival time of the NaCl treated male mice. The one female mouse that died prior to the 35 minute point survived for 13.3 minutes.

2.Effects of Beta Adrenergic Blockade on Physostigmine Protection:

Pretreatment with propranolol hydrochloride (10 mg/kg) 15 minutes prior to physostigmine did not alter the physostigmine protective effect against hypoxia, or the gender difference in protection (Fig. 1). Thus, as with physostigmine alone, a significantly ($P < 0.005$) greater percentage of female mice (100%, 10/10) compared to male mice (44%, 7/16) survived 35 minutes of hypoxia. The 9 propranolol-physostigmine treated male mice that did not

survive 35 minutes of hypoxia had a mean (± S.E.M.) survival time of 18.9 ± 2.1 minutes (Fig. 2), a value significantly ($p < 0.001$) greater than the NaCl treated control males (Fig. 2), but not significantly ($p > 0.05$) different from the male mice treated with physostigmine alone (17.1 ± 1.4 min).

3.Effect of Alpha Adrenergic Blockade on Physostigmine Protection:

a. Phentolamine. Pretreatment with phentolamine mesylate (4 mg/kg) 15 minutes before physostigmine, completely abolished the protective effect of physostigmine against hypoxia (Fig. 2). None of the 19 males treated with phentolamine-physostigmine and only 1 of 20 similarly treated females, survived 35 minutes of hypoxia. The mean (± S.E.M.) survival times of the phentolamine-physostigmine treated males (N=19) and females (N=19) were 3.9 ± 0.7 and 4.5 ± 0.6 minutes, respectively. These values were not significantly different from those of saline treated control mice. As a control, separate mice were treated with phentolamine alone 30 minutes prior to hypoxia. Their survival times were 2.4 ± 0.3 (N=6) and 2.2 ± 0.5 (N=9) minutes, for males and females respectively, both significantly ($p < 0.05$) shorter than the mean survival times of NaCl-treated mice.

b.Prazosin. Prazosin pretreatment also abolished physostigmine protection against hypoxia (Fig. 2). Thus, none of the 10 males or 9 females, given prazosin 4 mg/kg 15 minutes prior to physostigmine, survived 35 minutes of hypoxia. Indeed the survival times of these animals were extremely short, 1.8 ±

0.1 and 2.8 ± 0.6 minutes, respectively, for males and females. These survival times were significantly ($p<0.05$) shorter than those of NaCl-treated mice, but not significantly different from separate animals treated with prazosin alone (2.2 ± 0.2 minutes in 10 males, and 3.1 ± 0.5 minutes in 10 females).

Body Temperature :

The systemic injection of physostigmine reduces body temperature in mice (Maayani, et al., 1978; Minard and Grant, 1982; Saiyed and Riker, In Press). To examine the effect of propranolol on the hypothermia produced by physostigmine peritoneal implants of temperature-sensitive transmitters were made in 14 male mice. At least 15 days after implant these mice were treated with propranolol hydrochloride 10 mg/kg and 15 minutes later with physostigmine 0.2 mg/kg. Fifteen minutes after physostigmine injection the mice were introduced into the chamber and exposed either to flowing room air (N=4) or to the hypoxic gas mixture (5% O_2 - 95 % N_2) (N=10).

Figure 3 shows that under normoxic (room air) conditions pretreatment with propranolol did not alter the hypothermic effect of physostigmine in male mice, since the time course and the magnitude of the hypothermia was virtually identical to that of a control group of males pretreated with NaCl prior to physostigmine. As we observed previously during normoxia (Saiyed and Riker, In Press) the peak drop in body temperature occurred around 25 to 30 minutes and then gradually returned to near the control value at 75 minutes after physostigmine injection.

The time course of the change in body temperature of propranolol-physostigmine treated male mice (N = 10) during hypoxia is presented in figure 4. Five of the 10 male mice with implanted transmitters survived less than 35 minutes of hypoxia and the remaining 5 mice survived more than 35 minutes of

hypoxia. Body temperature continuously decreased during hypoxia, with no recovery phase as long as hypoxia persisted (Fig. 4). The rates and magnitudes of the body temperature changes in the short survivors (<< 35 minutes) were not significantly different than those of the long survivors (>> 35 minutes) at any time point.

Phentolamine pretreatment in 4 female mice did not alter the hypothermic effect of physostigmine during normoxia, as measured by rectal temperature. Figure 5 represents the time course of the changes in rectal temperature in female mice treated with phentolamine 15 minutes prior to physostigmine. As reported previously (Saiyed and Riker, In Press) the peak drop in body temperature occurs around 25 minutes and gradually returns to the control valve around 75 minutes after physostigmine injection. The maximum decrease in body temperature of -3.6 ± 0.8 °C (N = 4) was not different from the previously reported value of -3.7 ± 0.4 °C (N = 13) for female mice treated with physostigmine alone (Saiyed and Riker, In Press).

DISCUSSION

After the initial discovery by Scremin and Scremin (1979) that physostigmine protects male mice against hypoxia there have been repeated confirmations (Scremin and Scremin, 1980; Artru and Michenfelder, 1980; Minard and Grant, 1982; Riker, et al., 1990; Saiyed and Riker, In Press). However, the mechanism of physostigmine protection against hypoxia remains undefined. Originally, Scremin and Scremin (1979) proposed that an increase in cerebral blood flow caused by physostigmine was the probable mechanism of protection against hypoxia, but other authors (Artru and Michenfelder, 1980; Riker, et al., 1990) have proposed that physostigmine induced hypothermia may be an important element in protection.

It is generally accepted that hypothermia has cerebral protective effects (Kramer et al., 1968; Leonov et al., 1990), and such cerebral protection by hypothermia has been demonstrated in various experimental conditions including cardiac arrest (Kramer et al., 1968; Leonov et al., 1990), cerebral ischemia (Chopp et al., 1991), and hypoxia (Minard and Grant, 1982). The mechanism by which hypothermia protects the brain is not clear, although it has been attributed largely to a decrease in the cerebral metabolic rate of oxygen consumption ($CMRO_2$) (Carlsson et al., 1976; Michenfelder, 1988). Also, low ambient temperature, which facilitates the drop in body temperature during hypoxia, has been shown to prolong survival time of mice during hypoxia, whereas high ambient temperature, which reduces or prevents the drop in

body temperature, decreases survival during hypoxia (Artru and Michenfelder, 1981). In addition, the protective effect of physostigmine is significantly attenuated by preventing its hypothermic action (Artru and Michenfelder, 1980; Milde, 1988). While it therefore appears reasonable that protection by physostigmine is dependent on the drug induced hypothermia (Artru and Michenfelder, 1980; Minard and Grant, 1982) there are also several instances in which drug-induced hypothermia is not associated with protection against hypoxia. For example, neostigmine, chlorpromazine and reserpine all produce hypothermia in mice but do not protect against hypoxia (Minard and Grant, 1982; Saiyed and Riker, In Press). In the present study we have demonstrated a new dissociation of hypothermia and protection, in which phentolamine completely abolished the physostigmine protection without blocking its hypothermic effects. Thus physostigmine induced hypothermia alone is not the complete and sufficient explanation of its protective effect against hypoxia.

Systemic injection of physostigmine causes a widespread adrenergic activation, central in origin (Varagić and Kristić, 1966). As a consequence, physostigmine raises blood pressure (Dirnhuber and Cullumbine, 1955; Varagić, 1955; Medaković and Varagić, 1957; Kaul and Grewal, 1968), increases glycogenolysis in liver (Varagić, et al., 1967), and brain (Mršulja, et al., 1968), and also increases the output of epinephrine from the adrenal gland (Stewart and Rogoff, 1921; Kaul and Grewal, 1968). The pressor effect of physostigmine is mediated by alpha adrenergic receptors, whereas the

glycogenolytic effect is mediated by beta adrenergic receptors.

The increase in glycogenolysis after physostigmine is associated with an increase in blood glucose (Hrubetz, 1937; Varagić, et al., 1967). The hyperglycemic effect of physostigmine might be a factor in prolonging survival during hypoxia since hypoxia increases cerebral glucose utilization (Borgström, et al., 1976) and reduces cerebral glucose levels (Broniszewska-Ardelt and Jongkind, 1971; Norberg, et al., 1975). Furthermore, hyperglycemia increases the tolerance to hypoxia (Britton and Kline, 1945; Masukawa, et al., 1989), whereas hypoglycemia decreases tolerance (Britton and Kline, 1945). However, propranolol blocks the glycogenolytic effect of physostigmine (Varagić, et al., 1967; Mršulja, et al., 1968), but not the hypothermic (fig. 3) or the pressor effect (Varagić, et al., 1967; Kaul and Grewal, 1968). In the present study, propranolol did not alter the protective effect of physostigmine in male or female mice (fig. 1). Thus the physostigmine induced glycogenolysis and consequent hyperglycemia does not seem to be an essential or major factor in protection against hypoxia.

During normoxic conditions the cerebral blood flow (CBF) is autoregulated within a wide range of systemic blood pressure (Kontos, 1981; Busija and Heistad, 1984). However, in severe hypoxia the autoregulation of CBF is impaired and cerebral blood flow becomes pressure dependent (Freeman, 1968; Häggerdal, 1968; Busija and Heistad, 1984), so that a fall in blood pressure during hypoxia decreases cerebral blood flow (Busija and

Heistad, 1984). It is also known that hypoxia itself reduces blood pressure (Blood, et al., 1946; Marshall and Metcalfe, 1988), thereby decreasing cerebral oxygenation (MacMillan and Siesjö, 1971; Lewis, et al., 1973). Even a small decrease in the blood pressure creates a significant cerebral energy imbalance during hypoxia (Siesjö and Nilsson, 1971). Physostigmine, by increasing the blood pressure, would therefore help to maintain cerebral oxygenation and prevent changes in high energy phosphate levels during hypoxia. The pressor effect of physostigmine can be blocked by alpha adrenergic blocking drugs (Medaković and Varagić, 1957; Kaul and Grewal, 1968), and our present data shows that the protective effect of physostigmine against hypoxia is completely abolished by the alpha adrenergic blocking drugs, prazosin and phentolamine (fig. 2).

Phentolamine did not block the hypothermic effect of physostigmine during normoxia (fig. 5) or during hypoxia (unpublished observation), but these findings do not necessarily dismiss a protective role for hypothermia. Keykhah et al. (1982) reported that the cerebral protective effect of hypothermia against hypoxia is abolished if there is concurrent hypotension. An indication of this adverse effect of hypotension is seen in present study, in which the survival times of our animals treated with prazosin were significantly shorter than those of the saline treated mice. Although we did not monitor blood pressure it is probable that the doses of alpha adrenergic blockers we used not only blocked the pressor effect of physostigmine but also decreased the blood pressure

relative to the pretreatment level.

In summary we have examined the effects of pretreatment with alpha and beta adrenergic blocking drugs on the hypothermic and protective actions of physostigmine in male and female mice. The hypothermia induced by physostigmine was not blocked by either alpha or beta blockers. The protective effect of physostigmine against hypoxia was unaltered by the beta blocker propranolol, but completely abolished by the alpha blockers phentolamine and prazosin. We conclude that sympathetic activation by physostigmine plays an important role in protection against hypoxia, most likely by maintaining or elevating blood pressure. The results therefore support the previous work of others that maintenance or elevation of blood pressure during hypoxia is one of the critical factors in survival. Consequently, in the setting of normal or enhanced cardiovascular function, hypothermia induced by physostigmine may be the only additional factor needed for prolonged survival during severe hypoxia.

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Figure 1. Percentages of male and female mice surviving 35 minutes of hypoxia (5% O_2 - 95% N_2) after treatment with physostigmine (Phys) alone (left) or propranolol (Prpl) plus physostigmine (right). Mice in the Phys treatment group were injected with physostigmine, 0.2 mg/kg, 15 minutes prior to hypoxia. In the Prpl-Phys group propranolol, 10 mg/kg, was injected 15 min before physostigmine, 0.2 mg/kg, followed 15 min later by hypoxia exposure. Significant differences male vs female were $^{*}P<0.005$, $^{**}P<0.001$.

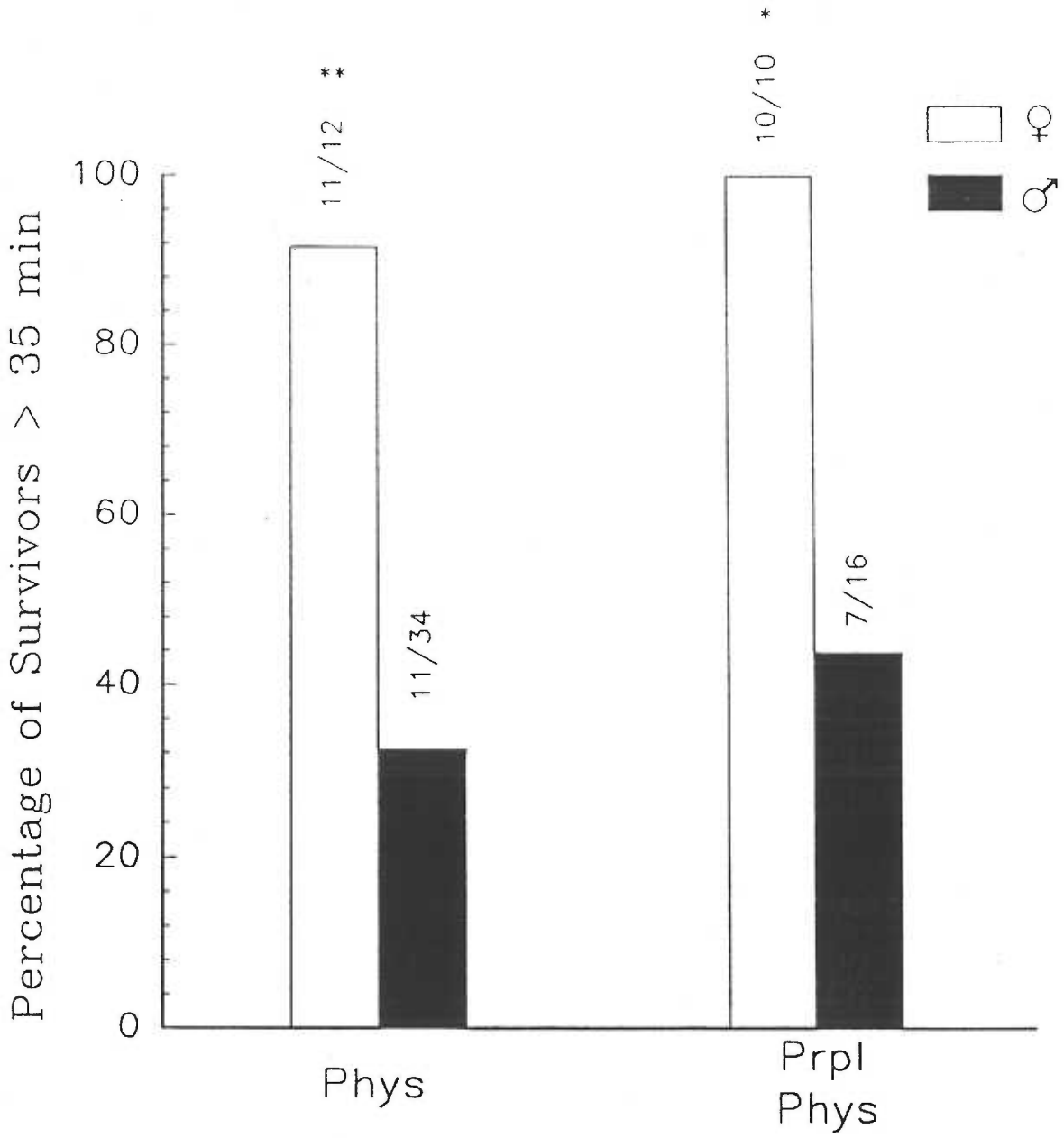
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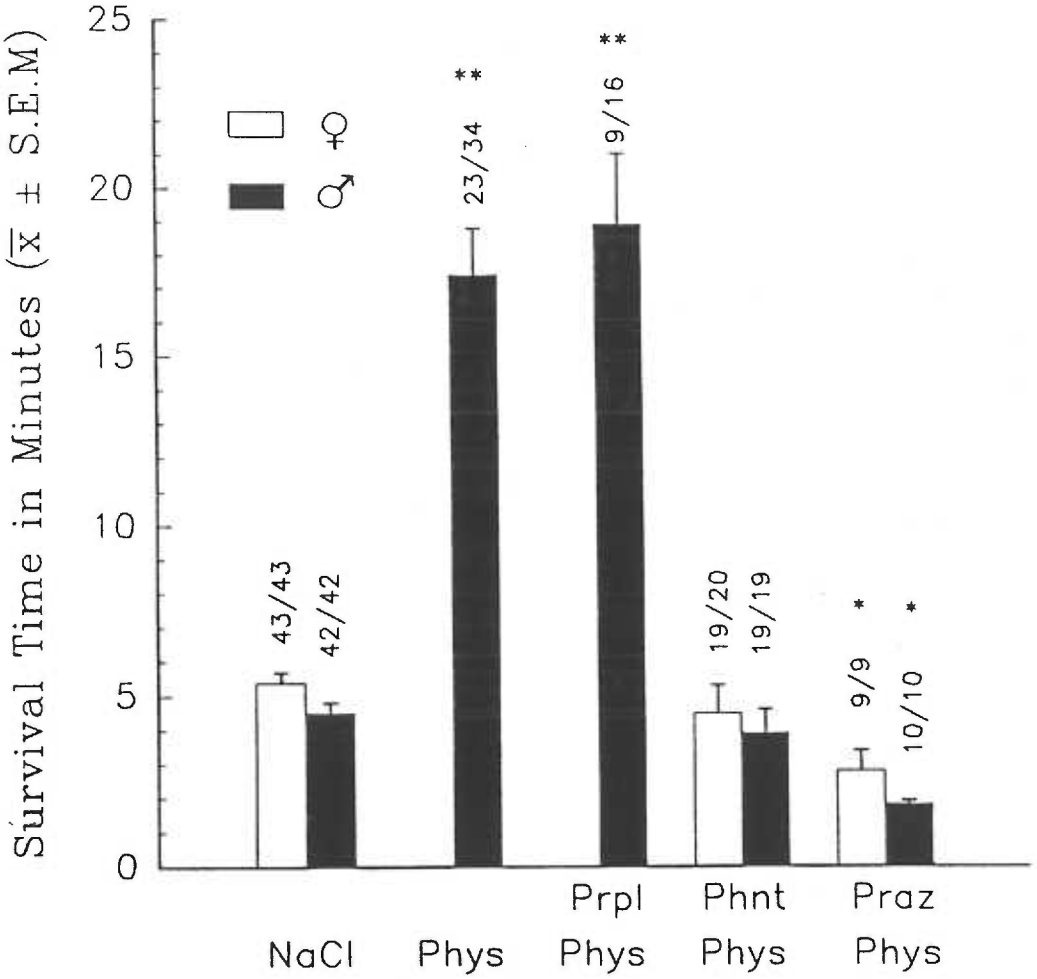
Figure 2. Survival times, in minutes ($\bar{x}$ ± S.E.M.), of male and female mice in different treatment groups surviving less than 35 minutes of hypoxia (5% O_2 - 95% N_2). The numbers above each bar represent the fraction of each treatment group that survived the mean time indicated on the ordinate. The remaining fraction of each group survived 35 minutes or longer. Mice were exposed to hypoxia 15 minutes after i.p. injection of either saline or physostigmine, 0.2 mg/kg. The adrenergic blocking drugs phentolamine, 4 mg/kg (Phys-Phnt), prazosin, 4 mg/kg (Phys-Praz) and propranolol, 10 mg/kg (Phys-Prpl) were injected i.p. 15 minutes prior to physostigmine, 0.2 mg/kg. Significant differences vs control (NaCl) were *$P<0.05$, **$P<0.001$. Note that no data for female mice is shown in the Phys and Phys-Prpl groups, since only one female in the Phys group and none in the Phys-Prpl group survived less than 35 minutes (see Fig. 1).


Survival Time in Minutes ($\bar{x}$ ± S.E.M)
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NaCl
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Prpl Phys
Phnt Phys
Praz Phys

Figure 3. Time course of decrease in body temperature after propranolol (10 mg/kg) and physostigmine (0.2 mg/kg) injection in 4 unrestrained male mice under normoxic conditions. Body temperature was measured remotely and continuously in these mice by peritoneally implanted temperature transmitters. Each value represents the $\bar{x}$ ± S.E.M. change in body temperature from the pretreatment control value. The times of injection of propranolol and physostigmine are indicated by arrows.

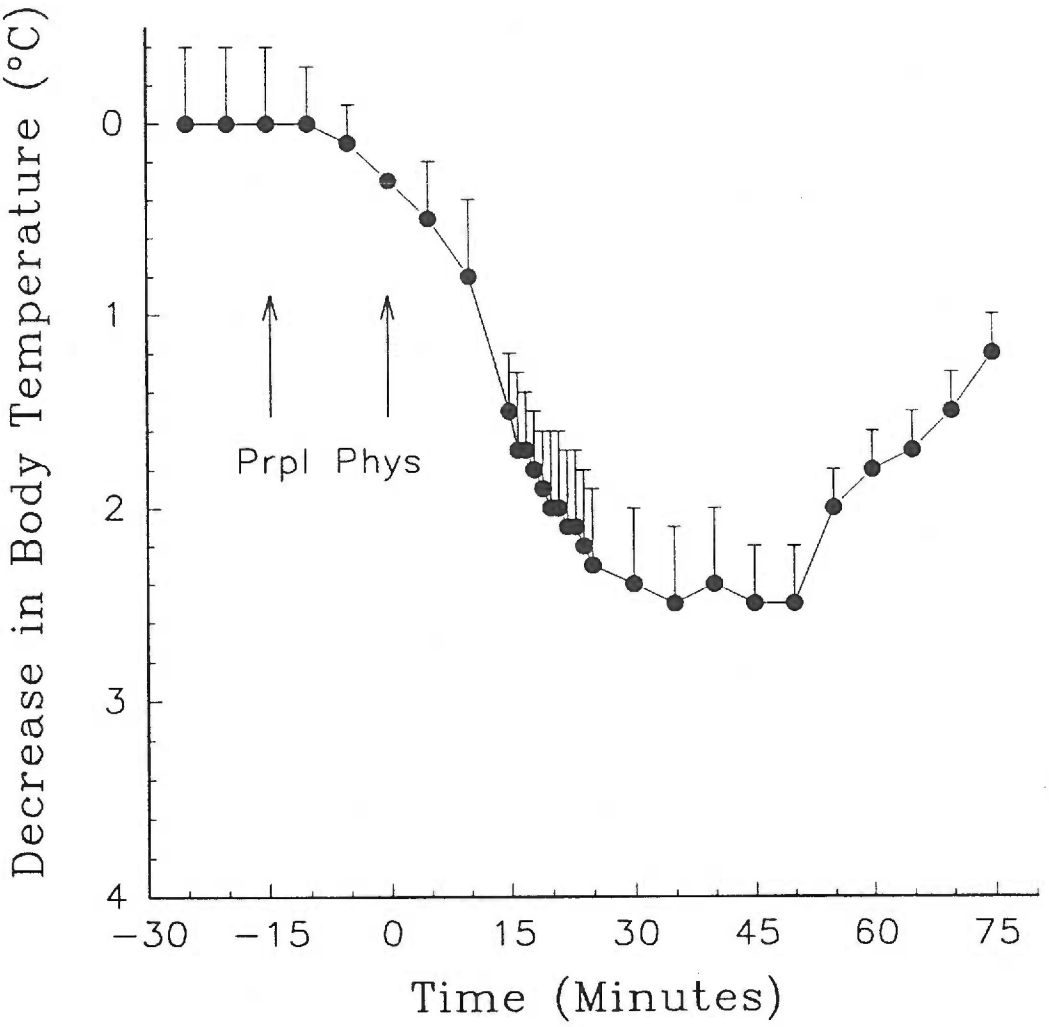

Decrease in Body Temperature (°C)
0
1
2
3
4
Prpl
Phys
−30
−15
0
15
30
45
60
75
Time (Minutes)

Figure 4. The time course of hypothermia during 60 minutes of hypoxia in 10 unrestrained male mice treated with propranolol 10 mg/kg (Prpl) and physostigmine 0.2 mg/kg (Phys). The body temperature was measured remotely and continuously before and during hypoxia by peritoneally implanted temperature transmitters. Arrows indicate the time of injection of propranolol (Prpl), physostigmine (Phys) and the onset of hypoxia. Each point is the $\bar{x}$ ± S.E.M.

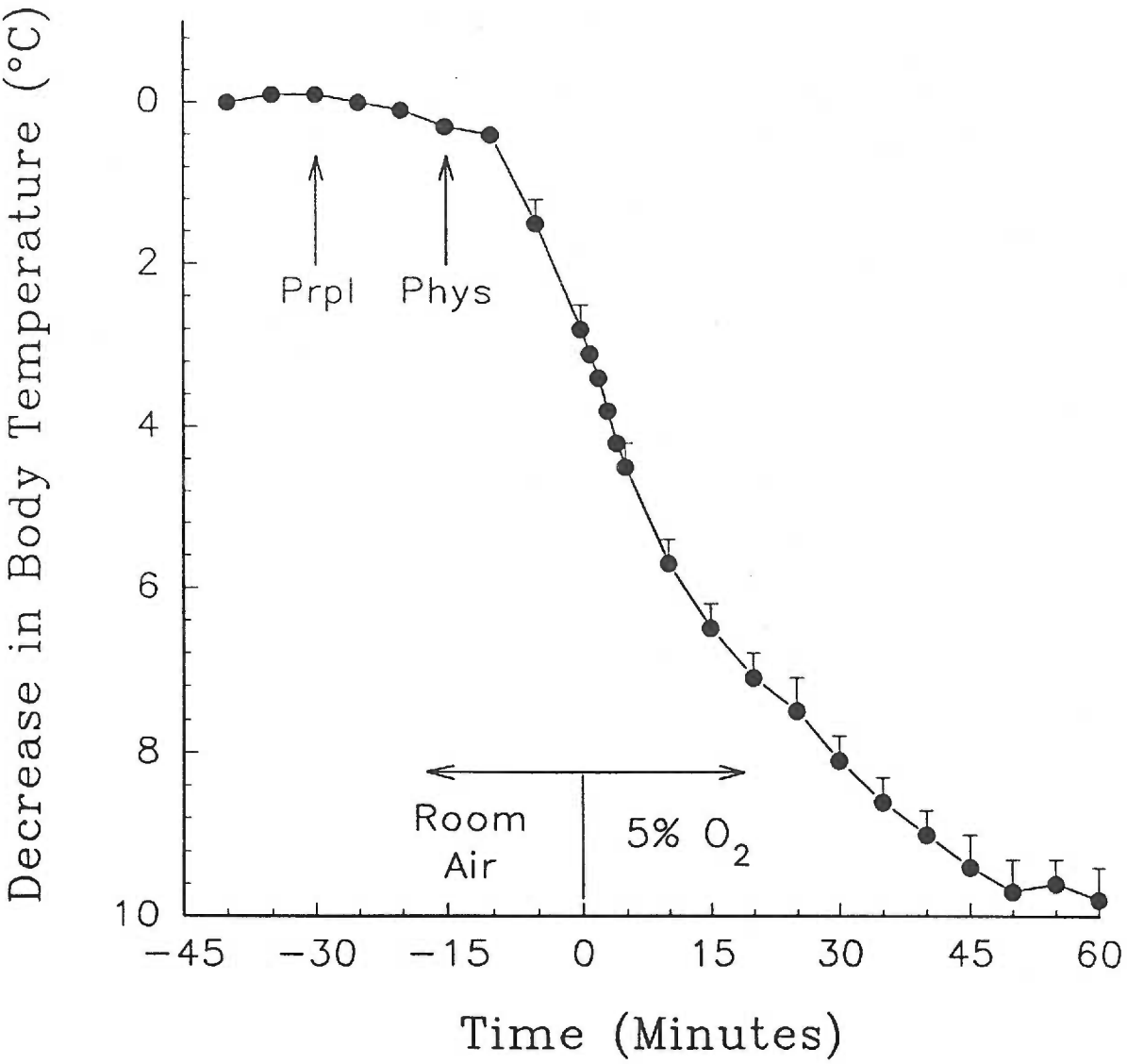
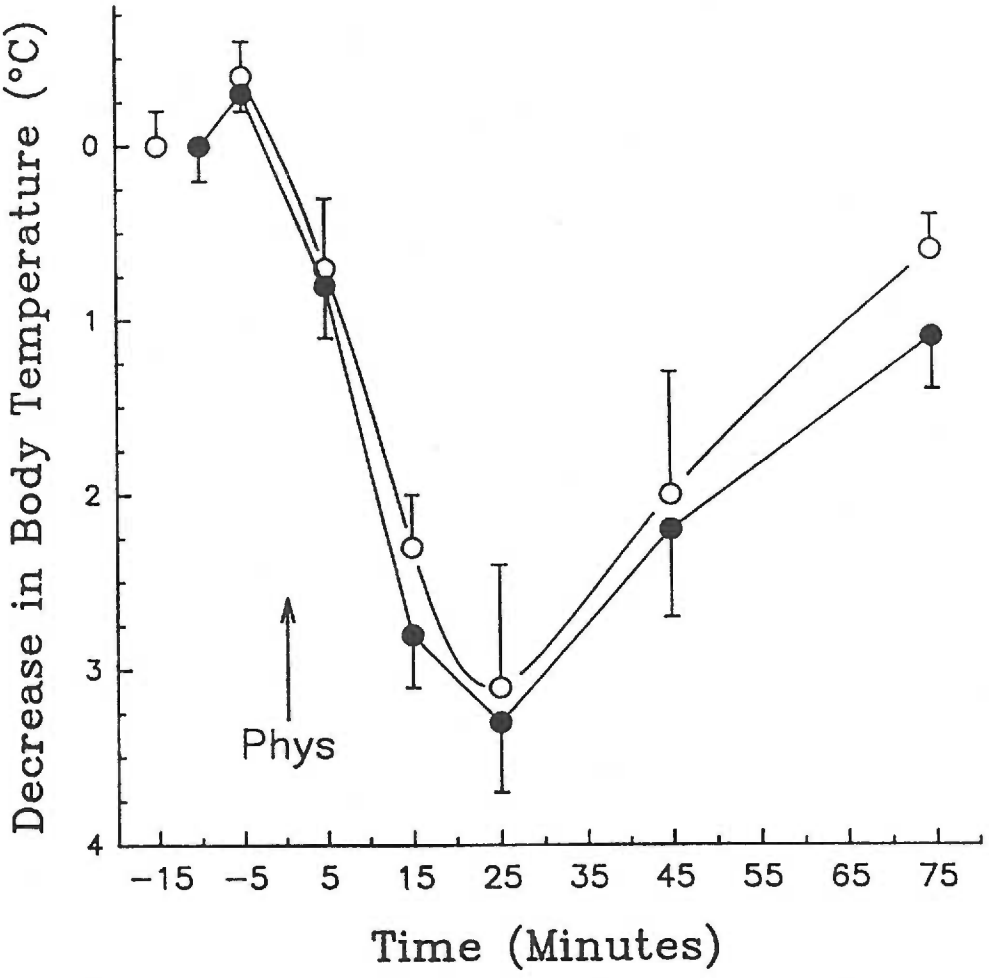

Decrease in Body Temperature (°C)
0
2
4
6
8
10
Prpl
Phys
Room Air
5% O_2
−45
−30
−15
0
15
30
45
60
Time (Minutes)

Figure 5. Time course of the hypothermia in female mice pretreated at -15 minutes with either i.p. NaCl (filled symbols, N=10), or with i.p. phentolamine, 4 mg/kg (open symbols, N=4) followed by i.p. physostigmine 0.2 mg/kg at time 0. Note that phentolamine pretreatment has no significant influence on physostigmine hypothermia. Each point is the $\bar{x}$ ± S.E.M.


Decrease in Body Temperature (°C)
0
1
2
3
4
Phys
−15
−5
5
15
25
35
45
55
65
75
Time (Minutes)

Discussion

The most important finding of the present investigation was the gender difference in physostigmine protection against hypoxia. Several studies have demonstrated that gender influences the outcome of cerebral ischemia, females being more resistant than males to neuronal damage from cerebral ischemia (Haberman, 1981; Rabinovitch et al., 1981; Payan and Conard, 1977; Yamori et al., 1976; McMurtry, 1973; Payan, 1970). Surprisingly, however, no substantial study was found on gender comparison in susceptibility to hypoxia or on drug protection against hypoxia. Indeed, in all of the published studies of drug protection against hypoxia in mice only one (reference 1 in Appendix) included females, and in that study the sexes were lumped together in the analysis of the data. Thus the present investigation is the first systematic study of gender comparison in susceptibility and in drug protection against acute hypoxia. The aim of the present investigation was to find (1) the mechanism of physostigmine protection and (2) the reason for the gender difference in protection.

As indicated in the introduction to this thesis, three actions of physostigmine were considered primarily important as possible mechanisms in protection against hypoxia: (1) hypothermia, (2) hyperglycemia and, (3) hypertension. The hypothermia induced by physostigmine is mediated by central cholinergic activation, whereas the pressor and hyperglycemic effects of physostigmine are produced by centrally initiated sympathoadrenal activation.

Hypothermia: No statistically significant gender difference was found in the hypothermic

effect of physostigmine (Manuscript I, II) when body temperatures were measured by the rectal probe method under normoxic conditions. The large variations in body temperature measurements might have masked possible gender differences. It is known that handling modifies body temperature in rodents. Therefore, peritoneally implanted temperature transmitters were used to avoid handling during body temperature measurements (Manuscript III).

The temperature measurements obtained by the transmitter implants showed that after physostigmine treatment female mice have a significantly lower body temperature than male mice. The gender difference in body temperature was statistically significant prior to the onset of hypoxia and this difference increased as hypoxia progressed (Manuscript III). The fact that females have a greater degree of hypothermia, and drop their temperature at a faster rate than do males may be a critical factor for the gender difference in physostigmine protection.

Whether hypothermia alone is sufficient as a mechanistic explanation for physostigmine's protective effect in hypoxia remains an open question. The finding that neostigmine produces a fall in body temperature but does not protect against hypoxia (Manuscript II), does not necessarily indicate that hypothermia alone cannot protect. The failure of neostigmine to protect mice against hypoxia may be related to the adverse effects of neostigmine on the cardiovascular and respiratory systems, i.e. hypotension and bronchoconstriction. The phenomenon of hypoxia-induced hypothermia has been known for a long time. Nevertheless, the mechanism of hypoxia-induced hypothermia is not known, although it is hypothesized that it is cholinergically mediated.

Future Directions for Hypothermia Research

1. Experiments are needed to test the possibility that the gender difference in degree and rate of hypothermia during hypoxia in physostigmine treated animals is the basis of the gender difference in physostigmine protection. Three possible types of experiments are suggested in the Discussion section of Manuscript III. Still another experimental approach would utilize the fact that mice can develop tolerance to the hypothermic effect of physostigmine after repeated administration. By producing a partial tolerance in females one might equalize their hypothermic response to that of the males.

2. To test the hypothesis that hypoxia-induced hypothermia is mediated by cholinergic activation, hypothermic responses to sublethal hypoxia could be examined after saline or atropine sulphate pretreatment. Atropine methylnitrate could also be used to differentiate whether the hypoxia-induced hypothermia originates centrally or peripherally, or from both sites.

Hyperglycemia: The finding that propranolol pretreatment did not alter the protective effect of physostigmine (Manuscript IV) leads to a tentative conclusion that physostigmine induced hyperglycemia may play no role, or only a minor one, in prolonging the survival of mice during hypoxia. However this conclusion can only be substantiated by measuring the blood and cerebral glucose levels in physostigmine and propranolol plus physostigmine treated animals during normoxia and hypoxia. Attempts were made during this thesis research to measure the cerebral glucose in mice, but we were not successful because of technical difficulty. Blood glucose levels were measured in the saline treated

and physostigmine treated mice during normoxia and hypoxia. It was found that in saline-treated animals the blood glucose decreased during hypoxia, whereas physostigmine treatment prevented the fall in blood glucose during hypoxia. Because of large variability in the glucose data it was not possible to conclude whether there was or was not a statistically significant gender difference in the hyperglycemic effect of physostigmine.

Future Directions for Hyperglycemia Research

1. Since measurements of blood glucose do not accurately reflect the cerebral glucose levels the latter should be measured in saline, physostigmine, and physostigmine plus propranolol treated animals, during normoxia and hypoxia. Ideally, such studies should be done with funnel-freezing of the brain in situ.

2. The gender difference observed (Manuscript I) in the persistence of cardiac contractions after respiratory arrest may be due to gender differences in the glycogen content of the heart. It has been reported that species with high cardiac glycogen levels tolerate hypoxia better than those with low glycogen concentration. Physostigmine has been shown to increase both glycogenolysis and cardiac glycogen levels in rats. No data exist for mice, however. An increase in cardiac glycogen might play a part in the ability to survive during hypoxia, and in the gender difference in survival. The mechanism responsible for accumulation of cardiac glycogen after physostigmine has not been described.

Hypertension: As indicated in the introduction to this thesis the maintenance of

blood pressure is very important during hypoxia. The two α-adrenergic blocking drugs used in the present investigation (phentolamine and prazosin) both blocked the protective effect of physostigmine, indicating that reduced blood pressure is incompatible with survival during hypoxia (Manuscript IV). The protective effect of hypothermia is also compromised by concomitant hypotension. Attempts were made during this thesis research to measure the mouse blood pressure by the tail cuff method, but these were not successful because of the small size of the animals. The smallest tail cuff equipment commercially available is intended for measurements in rats.

Future Directions for Hypertension Research

The measurement of blood pressure is the most important aspect which could not be accomplished in the present study. To solve the problem, a new method for measurement of blood pressure in mice needs to be developed. Alternatively, larger rodents, such as rats, could be used to measure the changes in blood pressure during hypoxia. The latter alternative would suffer from the fact that the hypothermic response of the rat to both physostigmine and hypoxia is highly variable and, when it occurs, less than that of the mouse.

The three preceding actions of physostigmine (hypothermia, hyperglycemia, hypertension) that were originally considered to be of primary importance in protection against hypoxia are obviously not the only ones that may be mechanistically important, particularly insofar as the gender difference is concerned. Of the many possible avenues of exploration, however, the following are suggested as essential.

Physostigmine Pharmacokinetics and Metabolism:

The thesis research provides indirect evidence that the gender difference in physostigmine protection is not attributable to a gender difference in the disposition of physostigmine after administration. Thus, the time courses of the hypothermic effect and of brain cholinesterase inhibition are not significantly different in male and female mice. Ideally, however, one would like to conclusively rule in or out whether there is a gender difference in physostigmine pharmacokinetics and/or metabolism.

Future Directions for Pharmacokinetic Research

Future research might include measurements of physostigmine levels in plasma and brain at various times after administration in both males and females. It should be noted, however, that even if this data were obtained there would remain the additional complicating question whether drug disposition during hypoxia develops a gender difference. Although the metabolizing activity of mixed-function oxidases is known to be depressed by hypoxia it is not known whether the depression differs according to sex.

Sex Steroids:

The observation that there was no gender difference in the survival times of the saline-treated control mice during hypoxia, yet there was a significant gender difference after physostigmine treatment suggests the possibility of a gender specific effect of physostigmine. As discussed briefly in MSII, this gender specific effect of physostigmine might be mediated through actions on the gonads (and/or pituitary) resulting in release

of sex steroids. The effect of physostigmine on sex steroid levels is not known. However, since estrogen is known to have an antihypoxic effect it was hypothesized that physostigmine may increase the estrogen level in the female, thus providing greater protection against hypoxia than that in males.

Future Directions for Sex Steroid Research

1. A comparison of the plasma levels of estrogen and testosterone in control and physostigmine treated animals could be the initial step for determining the effect of physostigmine.

2. If the hypothesis is correct that physostigmine increases the estrogen level in females and this is the basis of their longer survival then male mice injected with estrogen plus physostigmine should survive longer than male mice injected with physostigmine only.

3. Another approach would be to use gonadectomized animals. The absence of a gender difference in the antihypoxic effect of physostigmine in gonadectomized animals would strongly suggest the involvement of sex steroids in the mechanism of the gender difference. Furthermore, the gonadectomized male could be treated with estrogen and the gonadectomized female with testosterone to observe whether the gender difference in physostigmine protection was reversed.

Brain Oxygen Consumption and/or Utilization During Hypoxia:

The existing techniques for measurements of brain blood flow and oxygen

consumption are suited only to larger rodents and mammals, but not to mice. Therefore, it has not been possible to address perhaps the most important question: i.e., whether the gender difference in physostigmine protection ultimately depends on a significant gender difference in brain blood flow and/or oxygen consumption during hypoxia.

There is, however, an alternate approach to the question of oxygen consumption: namely, the measurement of brain mitochondrial respiration, specifically the rate of O_2 consumption during State 3 of mitochondrial respiration (for states of mitochondrial respiration see Zubay, 1983). Some years ago it was shown (Mela, 1979, 1982) that brain, liver and heart mitochondrial preparations isolated from male rats previously exposed to severe hypoxia (7% O_2) exhibited very large increases (50-100%) in State 3 respiration rates. Thus, these hypoxia-induced changes in mitochondrial function occurred only in-vivo and persisted in the isolated mitochondria in vitro for some finite time (at least an hour) after hypoxia exposure. This response was not due to uncoupling since State 4 respiratory activities were very low. Rather, the data indicate that within minutes after the start of hypoxia (PO_2 = 30 mm Hg) there is a greatly increased efficiency in mitochondrial respiration, hence in synthesis of ATP.

We have made preliminary studies of this phenomenon utilizing crude synaptosomal fractions isolated from male and female mouse brains. In these experiments we compared groups of physostigmine-treated normoxic and hypoxic (5% O_2) mice (3-8 animals per group). The mice were treated with 0.2 mg/kg physostigmine, i.p., then 15 minutes later were exposed to hypoxia or left in room air. They were then sacrificed at either 10 minutes or 25 minutes after the start of hypoxic (or room air)

exposure. The results demonstrated that in mice, as in rats (Mela, 1979, 1982), hypoxia induced a significant increase in State 3 respiration rates in brain mitochondria, compared to the rates in normoxic mice.

The most remarkable feature of this preliminary work is that the hypoxia-induced increase in State 3 respiration rate occurred 10 minutes after the start of hypoxia in females, but not in males. The male increase was present, however, at 25 minutes. Consequently, it appears that brain mitochondria in females adapt to low oxygen in-vivo much more rapidly than do the brain mitochondria in males. Pursuit of this important finding, with larger numbers of animals and greater definition of the time course should be the first step in future research on the gender difference in physostigmine protection against hypoxia. At the same time it would be essential to try and explain the as yet unidentified mechanism(s) of this hypoxia-induced change in mitochondrial function.

Conclusion

The present thesis research has demonstrated, for the first time, a significant gender difference in the protective effect of physostigmine against otherwise lethal hypoxia. In addition, the work embodied in manuscripts I-IV has helped to clarify some of the mechanistic components of physostigmine protection, notably the importance of central cholinergic activation including the resulting hypothermia, and the essential role of the α adrenergic system. The gender difference in the hypothermic responses to physostigmine and hypoxia may be at least a partial explanation for the gender difference in the survival of physostigmine-treated mice during hypoxia.

The broader significance of this thesis research may relate to the observed phenomena that appear to be drug specific. For example, the following APPENDIX shows that an extremely wide spectrum of drugs can prolong survival of mice during hypoxia. However, all except physostigmine prolong survival only by minutes. Physostigmine, in contrast to all the others, is the only drug that enables some fraction of treated mice to survive at least a full hour of hypoxia (reference 6 in APPENDIX). We now know, from the present research, that a significantly greater percentage of females, compared to males, survive one hour of hypoxia. Thus, both long term survival and the gender difference are, at least so far, unique to physostigmine. Considering the widespread use of physostigmine in post-anesthetic recovery and in the treatment of Alzheimer's disease the thesis research raises the question whether gender difference may also be present in these therapeutic applications of physostigmine.

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APPENDIX

Table 1: Selective Summary of Relevant Published Literature Concerning Pharmacological Protection of Mice Exposed to Hypoxia (1962 - 1988).

Drug / Dose	Interval (min) between drug administration and hypoxia	Sex / Strain / Body Wt. (g)	Hypoxia Chamber Conditions			Survival Times (min)		% Change in Survival Time	Ref.
			% O_2	Flow L/min	Temp °C	Control	Treated		
Thiopental 52-87 mg/kg	5-30	Male/Female Wistar 20-30	0-7	Not given	24	3*	45*	≈1400	1
Ether 4-6%	10	Male White 25-30	5	6	32-34	3.1	3.1	0	2
Chloroform 1-2%	10	Male White 25-30	5	6	32-34	3.6	4.0	11	2
Cyclopropane 25-30%	10	Male White 25-30	5	6	32-34	2.9	4.1	41	2
Halothane 1-2%	10	Male White 25-30	5	6	32-34	2.7	5.0	85	2

Table 1: Selective Summary of Relevant Published Literature Concerning Pharmacological Protection of Mice Exposed to Hypoxia (1962 - 1988).

Drug / Dose	Interval (min) between drug administration and hypoxia	Sex / Strain / Body Wt. (g)	Hypoxia Chamber Conditions			Survival Times (min)		% Change in Survival Time	Ref.
			% O_2	Flow L/min	Temp °C	Control	Treated		
Methoxyflurane 0.5-1.5%	15	Male White 25-30	5	6	32-34	2.9	3.3	14	2
Nitrous Oxide 20-95%	10	Male White 25-30	5	6	32-34	2.8	2.5	-11	2
Thiopental 1.8 mg/kg	5-10	Male White 25-30	5	6	32-34	3.1	8.0	158	2
Thiopental 1.5-1.8 mg + Halothane 1%	10	Male White 25-30	5	6	32-34	3.0	4.7	57	3
Urethane 50 mg	12-13	Male White 25-30	5	6	32-34	2.6	4.6	77	3

Table 1: Selective Summary of Relevant Published Literature Concerning Pharmacological Protection of Mice Exposed to Hypoxia (1962 - 1988).

Drug / Dose	Interval (min) between drug administration and hypoxia	Sex / Strain / Body Wt. (g)	Hypoxia Chamber Conditions			Survival Times (min)		% Change in Survival Time	Ref.
			% O_2	Flow L/min	Temp °C	Control	Treated		
Hydroxydione 1 mg	12-13	Male White 25-30	5	6	32-34	2.4	4.6	92	3
Detrovel 4 mg	12-13	Male White 25-30	5	6	32-34	2.5	3.8	52	3
Methohexital Dose[a]	25-30	Male White 25-40	5	6	32-34	4.3±0.2	4.2±0.5	-2	4
Barbital Dose[a]	25-30	Male White 25-40	5	6	32-34	4.6±0.3	5.0±0.4	9	4
Phenobarbital Dose[a]	25-30	Male White 25-40	5	6	32-34	4.4±0.2	6.4±0.4	45	4
Amylobarbital Dose[a]	25-30	Male White 25-40	5	6	32-34	4.8±0.3	7.5±0.8	56	4

Table 1: Selective Summary of Relevant Published Literature Concerning Pharmacological Protection of Mice Exposed to Hypoxia (1962 - 1988).

Drug / Dose	Interval (min) between drug administration and hypoxia	Sex / Strain / Body Wt. (g)	Hypoxia Chamber Conditions			Survival Times (min)		% Change in Survival Time	Ref.
			% O_2	Flow L/min	Temp °C	Control	Treated		
Allypropymal Dose[a]	25-30	Male White 25-40	5	6	32-34	4.9±0.2	10±0.8	104	4
Hexobarbital Dose[a]	25-30	Male White 25-40	5	6	32-34	5.2±0.4	12.5±1.3	140	4
Pentobarbital Dose[a]	25-30	Male White 25-40	5	6	32-34	5.0±0.3	14.4±1.9	188	4
Pentobarbital 25 mg/kg	30	Male Sprague Dawley 19-33	5	Not given	32-34	3.8±0.2	5.2±1.0	37	5
Pentobarbital 30 mg/kg	30	Male Sprague Dawley 19-33	5	Not given	32-34	3.8±0.2	4.6±0.7	21	5

Table 1: Selective Summary of Relevant Published Literature Concerning Pharmacological Protection of Mice Exposed to Hypoxia (1962 - 1988).									
Drug / Dose	Interval (min) between drug administration and hypoxia	Sex / Strain / Body Wt. (g)	Hypoxia Chamber Conditions			Survival Times (min)		% Change in Survival Time	Ref.
			% O_2	Flow L/min	Temp °C	Control	Treated		
Pentobarbital 35 mg/kg	30	Male Sprague Dawley 19-33	5	Not given	32-34	3.8±0.2	7.5±0.9	97	5
Pentobarbital 40 mg/kg	30	Male Sprague Dawley 19-33	5	Not given	32-34	3.8±0.2	9.1±1.1	139	5
Pentobarbital 50 mg/kg	30	Male Sprague Dawley 19-33	5	Not given	32-34	3.8±0.2	14.2±1.4	274	5
Pentobarbital 60 mg/kg	30	Male Sprague Dawley 19-33	5	Not given	32-34	3.8±0.2	15.3±1.9	303	5

Table 1: Selective Summary of Relevant Published Literature Concerning Pharmacological Protection of Mice Exposed to Hypoxia (1962 - 1988).

Drug / Dose	Interval (min) between drug administration and hypoxia	Sex / Strain / Body Wt. (g)	Hypoxia Chamber Conditions			Survival Times (min)		% Change in Survival Time	Ref.
			% O_2	Flow L/min	Temp °C	Control	Treated		
Pentobarbital 70 mg/kg	30	Male Sprague Dawley 19-33	5	Not given	32-34	3.8±0.2	14.2±1.9	274	5
Pentobarbital 90 mg/kg	30	Male Sprague Dawley 19-33	5	Not given	32-34	3.8±0.2	4.6±0.7	21	5
Pentobarbital 100 mg/kg	30	Male Sprague Dawley 19-33	5	Not given	32-34	3.8±0.2	2.1±0.3	-18	5
Chlorpromazine 25 mg/kg	30	Male Sprague Dawley 19-33	5	Not given	32-34	3.8±0.2	2.9±0.4	-24	5
Chlorpromazine 25 mg/kg	30	Male Sprague Dawley 19-33	5	Not given	-20	2.9±0.4	13.6±1.5	369	5

Table 1: Selective Summary of Relevant Published Literature Concerning Pharmacological Protection of Mice Exposed to Hypoxia (1962 - 1988).

Drug / Dose	Interval (min) between drug administration and hypoxia	Sex / Strain / Body Wt. (g)	Hypoxia Chamber Conditions			Survival Times (min)		% Change in Survival Time	Ref.
			% O_2	Flow L/min	Temp °C	Control	Treated		
Physostigmine 0.1 mg/kg	15-20	Male White 20-25	5	5	25±0.5	4.3±0.4	6.7±.9	56 (2/14 survived more than one hour)	6
Physostigmine 0.2 mg/kg	15-20	Male White 20-25	5	5	25±0.5	4.3±0.4	13.9±1.6	223 (4/14 survived more than one hour)	6
Physostigmine 0.3 mg/kg	15-20	Male White 20-25	5	5	25±0.5	4.3±0.4	27.6±4.4	542 (4/14 survived more than one hour)	6

Table 1: Selective Summary of Relevant Published Literature Concerning Pharmacological Protection of Mice Exposed to Hypoxia (1962 - 1988).

Drug / Dose	Interval (min) between drug administration and hypoxia	Sex / Strain / Body Wt. (g)	Hypoxia Chamber Conditions			Survival Times (min)		% Change in Survival Time	Ref.
			% O_2	Flow L/min	Temp °C	Control	Treated		
Pentobarbital 50 mg/kg	23	Male ARS HA/ICR Albino 24-33	5-5.25	3.5-4	35	3.9±0.1	14.0±0.8	259	7
Physostigmine 0.1 mg/kg	20	Male ARS HA/ICR Albino	5	3.5-4	35	3.9±0.1	5.0±0.4	28	7
Physostigmine 0.2 mg/kg	20	Male ARS HA/ICR Albino	5	3.5-4	35	3.9±0.1	5.0±0.4	28	7
Physostigmine 0.3 mg/kg	20	Male ARS HA/ICR Albino	5	3.5-4	35	3.9±0.1	6.3±0.3	62	7

Table 1: Selective Summary of Relevant Published Literature Concerning Pharmacological Protection of Mice Exposed to Hypoxia (1962 - 1988).

Drug / Dose	Interval (min) between drug administration and hypoxia	Sex / Strain / Body Wt. (g)	Hypoxia Chamber Conditions			Survival Times (min)		% Change in Survival Time	Ref.
			% O_2	Flow L/min	Temp °C	Control	Treated		
PGI_2 1 mg/kg	30	Male std-ddy 22-24	4	4	Not given	3.3*	7.3*	121	8
PGE_1 3 mg/kg	30	Male std-ddy 22-24	4	4	Not given	3.3*	7.8*	136	8
PGD_2 10 mg/kg	30	Male std-ddy 22-24	4	4	Not given	3.3*	6.8*	106	8
Physostigmine 0.1 mg/kg	30	Male ICR 25	4	5	Not given	3.5*	9.5*	171	9
Dihydroergotoxin 1 mg/kg	30	Male ICR 25	4	5	Not given	3.3*	2.4*	-27	9
Indeloxazine 10 mg/kg	30	Male ICR 25	4	Not given	Not given	3.3*	6.6*	100	9

Table 1: Selective Summary of Relevant Published Literature Concerning Pharmacological Protection of Mice Exposed to Hypoxia (1962 - 1988).

Drug / Dose	Interval (min) between drug administration and hypoxia	Sex / Strain / Body Wt. (g)	Hypoxia Chamber Conditions			Survival Times (min)		% Change in Survival Time	Ref.
			% O_2	Flow L/min	Temp °C	Control	Treated		
Thiopental 100 mg/kg	20	Male Swiss White 26-43	5	3	25	4.8±0.4	11.8±2.2	146	10
Thiopental 100 mg/kg	20	Male Swiss White 26-43	5	3	35.5	5.0±0.3	4.3±0.4	-14	10

[a]Doses adjusted to be equimolar with 55 mg/kg thiopental.
*Values estimated from figure.

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