Threshold levels of maternal nicotine impairing protective responses of newborn rats to intermittent hypoxia

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Fewell, James E., Francine G. Smith, and Vienna K. Y. Ng. Threshold levels of maternal nicotine impairing protective responses of newborn rats to intermittent hypoxia. J Appl Physiol 90: 1968–1976, 2001.—Experiments were carried out to determine the threshold level of maternal nicotine that impairs protective responses of rat pups to hypoxia. From days 6 or 7 of gestation, pregnant rats received either vehicle or nicotine (1.50, 3.00, or 6.00 mg of nicotine tartrate·kg body wt (−1·day−1)) or vehicle continuously via a subcutaneous osmotic minipump. On postnatal days 5 or 6, pups were exposed to a single period of hypoxia produced by breathing an anoxic gas mixture (97% N2 or 3% CO2) and their time to last gasp was determined, or they were exposed to intermittent hypoxia and their ability to autoresuscitate from hypoxic-induced primary apnea was determined. Perinatal exposure to nicotine did not alter the time to last gasp or the total number of gasps when the pups were exposed to a single period of hypoxia. The number of successful autoresuscitations on repeated exposure to hypoxia was, however, decreased in pups whose dams had received either 3.00 or 6.00 mg of nicotine tartrate·kg body wt; these dosage regimens produced maternal serum nicotine concentrations of 19 ± 6 and 35 ± 8 ng/ml, respectively. Thus our experiments define the threshold level of maternal nicotine that significantly impairs protective responses of 5- to 6-day-old rat pups to intermittent hypoxia such as may occur in human infants during episodes of prolonged sleep apnea or positional asphyxia.

apnea; hypoxic gasping; perinatal drug exposure; sudden infant death syndrome

MATERNAL CIGARETTE SMOKING increases the risk of fetal and neonatal death as well as various complications of pregnancy, including fetal growth retardation, spontaneous abortion, abruptio placenta, placenta previa, and premature birth (24). In addition, maternal cigarette smoking is a major and independent risk factor for sudden infant death syndrome (SIDS) (6, 19, 20, 29–31, 33, 34, 43, 45); the risk increases in proportion to the number of cigarettes smoked (19). Smoking more than 20 cigarettes a day, or heavy smoking, increases the relative risk of SIDS fivefold when compared with nonsmokers. In the National Institute of Child Health and Human Development study of epidemiological factors for SIDS (20), the relative risk for SIDS associated with maternal cigarette smoking was 3.4; this was higher than any other maternal or newborn condition evaluated, with a frequency of smoking among SIDS mothers of 70%. Despite these well-known risks, ~25% of women in the United States continue to smoke cigarettes during pregnancy (1, 8).

Cigarette smoke contains a wide variety of chemicals, including nicotine (57), which easily crosses the placenta and is found in placental tissue, in amniotic fluid, and in fetal cord blood in concentrations equal to or greater than those measured in maternal blood (27, 28, 54). We have previously shown that perinatal exposure to nicotine impairs the ability of 5- to 6-day-old rat pups to “autoresuscitate” from intermittent hypoxic-induced primary apnea (11). This is important because autoresuscitation failure from hypoxic-induced primary apnea, whether caused by prolonged sleep apnea or positional asphyxia, has been hypothesized as a final event leading to sudden death in some infants (21, 22). In our previous study, we used a relatively high dose of nicotine (i.e., 6 mg of nicotine tartrate·kg maternal body wt (−1·day−1)) to achieve maternal serum nicotine levels comparable to those observed in heavy smokers (i.e., 30–40 ng/ml; Ref. 4). The question arises, however, as to whether lower maternal serum nicotine levels, similar to those observed with the use of the nicotine patch, which has been advocated for use in pregnant women who cannot stop smoking with behavioral treatment alone (3), would also result in impairment of the aforementioned protective responses of the newborn to hypoxia. Accordingly, the present experiments were carried out to determine the threshold level of maternal nicotine that impairs protective responses of newborn rats to hypoxia.

METHODS

Fifty-four pregnant Sprague-Dawley rats undergoing their first pregnancy and 61 rat pups born by spontaneous vaginal delivery at term of gestation were studied. Adult rats were...
housed individually in Plexiglas cages and had continuous access to food (Lab Diet 5001) and tap water. Pups were housed with their mother and siblings until study. All animals were housed at 22 ± 1°C in a 12:12-h light-dark cycle (lights on at 0700).

**Surgical Preparation**

The pregnant rats underwent one operation on day 6 or 7 of gestation when they were anesthetized by inhalation of halothane (−2.0% for induction and maintenance) in oxygen and placed in a prone position. A small incision was made over the scapulae, and a 28-day osmotic minipump (2ML4, ALZET) was inserted subcutaneously for continuous infusion of nicotine tartrate or vehicle. In this species, implantation of the embryo in the uterine wall begins on day 5 and is complete on day 7 (9).

All surgical and experimental procedures were carried out in accordance with the Guide to the Care and Use of Experimental Animals provided by the Canadian Council on Animal Care and with the approval of the Animal Care Committee of the University of Calgary.

**Experimental Protocols**

**Experiment I:** maternal serum nicotine concentrations. Pregnant rats that received either 0, 0.75, 1.50, 2.25, 3.00, or 6.00 mg of nicotine tartrate−kg body wt−1·day−1 from day 6 or 7 of gestation were anesthetized on day 10 (n = 3 at each dose), day 15 (n = 3 at each dose), or day 20 (n = 3 at each dose) of gestation; blood was immediately obtained via cardiac puncture for later determination of serum nicotine concentration.

**Experiment II:** time to last gasp during a single anoxic gas challenge. For an experiment, each 5- to 6-day-old pup whose mother had received either 0 (n = 8), 1.50 (n = 8), 3.00 (n = 8), or 6.00 (n = 8) mg of nicotine tartrate−kg body wt−1·day−1 from day 6 or 7 of gestation was weighed and placed into a metabolic chamber regulated at 37.0 ± 0.1°C into which flowed room air at a rate of 1 l/min. At the end of a 30-min stabilization period, the gas that flowed into the chamber was changed from room air to 97% N2 and 3% CO2 and the time to last gasp was determined. At the beginning of the hypoxic exposure, the chamber was flushed with the anoxic gas mixture until the gas concentrations in the chamber had stabilized; the flow rate was then lowered to 1 l/min. During a time-to-last-gasp experiment, the stages of the respiratory response to hypoxia as well as the time to last gasp were directly observed on the polygraph tracing. The respiratory response of both newborn (25) and adult (18) animals to acute hypoxia typically passes through four stages: hyperpnea, primary apnea, gasping, and terminal apnea.

**Experiment III:** autoresuscitation from primary apnea during repeated anoxic gas challenges. For an experiment, each 5- to 6-day-old pup whose mother had received either 0 (n = 8), 1.50 (n = 8), 3.00 (n = 5), or 6.00 (n = 8) mg of nicotine tartrate−kg body wt−1·day−1 from day 6 or 7 of gestation was weighed and placed into a metabolic chamber regulated at 37.0 ± 0.1°C into which flowed room air at a rate of 1 l/min. At the end of a 30-min stabilization period, the gas that flowed into the metabolic chamber was changed from room air to 97% N2 and 3% CO2 until primary apnea occurred; the gas was then changed back to room air, and the ability of the pup to autoresuscitate by gasping was determined. This procedure was repeated at 5-min intervals from the start of the first anoxic gas challenge until death occurred. Again, when the gas mixture was changed, the flow rate was increased until the gas concentrations in the chamber had stabilized; the flow rate was then lowered to 1 l/min. During an experiment, primary apnea was detected by directly observing the absence of respiratory movements on the polygraph tracing.

**Experimental Apparatus**

The metabolic chamber used in our experiments consisted of a double-walled Plexiglas cylinder (30 cm long, internal diameter of 6 cm) into which flowed room air or 97% N2 and 3% CO2. Chamber ambient temperature was controlled to 37.0 ± 0.1°C by circulating water from a temperature-controlled bath (Neslab, endocal refrigerated circulating bath RTE-SDD) through the space between the walls.

**Experimental Measurements and Calculations**

During an experiment, the electrocardiogram, respiratory movements, and chamber CO2 levels were recorded on a model 7 polygraph (Grass Instruments) at a paper speed of 10 mm/s. A bipolar lead II electrocardiogram was recorded from multistranded stainless steel wire electrodes (AS 633, Cooner Wire) sewn on the right shoulder (− electrode) and the left thigh (+ electrode) as described by Osborne (37); the electrodes were connected to a model 7HIP5 high-impedance probe coupled to a model 7P5 wide-band electroencephalogram AC preamplifier (Grass Instruments). Respiratory movements were recorded from a mercury-in-silicone rubber strain gauge (model HgPC, DM Davis) placed around the chest; the strain gauge was connected to a bridge amplifier (Biomedical Technical Support Center, University of Calgary) that was coupled to a model 7P03 adapter panel (Grass Instruments). Chamber CO2 levels were measured using an applied electrochemistry CO2 analyzer (Ametek) coupled to a model 7P03 adapter panel.

**Nicotine**

Nicotine (hydrogen tartrate salt, [−]-nicotine di-[+]-tartrate salt, Sigma Chemical) was dissolved in sterile water and infused at a rate of 60 μl/day to give doses of 0.75, 1.50, 2.25, 3.00, or 6.00 mg of nicotine tartrate−kg maternal body wt−1·day−1 from day 6 or 7 of gestation based on a final average body weight of 330 g. Sterile water was used as vehicle. Serum concentrations of nicotine were determined by gas chromatography-mass spectrometry using the selected ion monitoring mode (Centre for Toxicology, University of Calgary).

**Statistical Analysis**

Statistical analysis was carried out using an ANOVA followed by a Newman-Keuls multiple-comparison test to determine whether dose of nicotine tartrate affected body weight, basal respiratory and heart rates, time to last gasp, total number of gasps, and the number of successful autoresuscitations. All results are reported as means ± SD, and P < 0.05 was considered to be of statistical significance.

**RESULTS**

**Maternal Serum Nicotine Concentrations**

The maternal serum nicotine concentrations in dams that received 0–6.00 mg of nicotine tartrate/kg body wt over each 24-h period from day 6 or 7 of gestation are shown in Fig. 1. On day 20 of gestation, fetal serum nicotine concentrations ranged from 91 to 240% of maternal serum nicotine concentrations.
Body Weight and Basal Respiratory Rate and Heart Rate

Perinatal exposure to nicotine in doses up to 6.00 mg of nicotine tartrate/kg of body weight did not significantly alter the body weight, basal heart rate, or basal respiratory rate in 5- to 6-day-old pups compared with that observed in pups that received vehicle during the perinatal period (Fig. 2).

Experiment I: Time to Last Gasp During a Single Anoxic Gas Challenge

Exposure to a single period of hypoxia resulted in a reproducible respiratory response in all pups. The respiratory response consisted of hyperpnea, primary apnea, gasping, and terminal apnea; in all animals, terminal apnea preceded the appearance of arrhythmias or an isoelectric pattern on the electrocardiogram. Gasping occurred in three phases: an initial phase of rapid gasping (phase I), followed by a period of slower gasping (phase II), and finally a period of rapid gasping (phase III) that eventually waned and gave way to terminal apnea and death. Perinatal exposure to nicotine did not significantly alter the aforementioned gasping patterns of pups compared with those observed in pups exposed to vehicle during the perinatal period. Furthermore, perinatal exposure to nicotine did not alter the time to last gasp or the total number of gasps during a single anoxic gas challenge (Fig. 3).

Experiment II: Autoresuscitation From Primary Apnea During Repeated Anoxic Gas Challenges

Repeated exposure to hypoxia elicited similar cardiorespiratory and arousal patterns in all successful autoresuscitations (Fig. 4). Initially, there was a period of hyperpnea (Fig. 4A) and arousal (Fig. 4B) that preceded primary apnea and bradycardia (Fig. 4C). During the arousal phase, all animals were awake and exhibited pronounced locomotor activity in an apparent attempt to “escape” the anoxic environment. Tonic
posturing with the neck and back arched and extremities extended (opisthotonus) appeared before or with the onset of primary apnea. During primary apnea, the pups became limp and unresponsive to somatosensory stimuli (i.e., tugging on a suture attached to the skin of the back) before the onset of gasping. Gasping (Fig. 4D) was followed by an increase in heart rate (i.e., cardiac resuscitation) and then restoration of a normal respiratory pattern (Fig. 4E) (i.e., respiratory resuscitation).

Perinatal exposure to nicotine impaired the ability of 5- to 6-day-old pups to autoresuscitate from hypoxic-induced primary apnea in a dose-dependent manner (Fig. 5). The sequence of events leading to autoresuscitation failure was influenced by the level of nicotine exposure during the perinatal period (Table 1). In six of seven pups that were exposed to vehicle and five of seven pups that were exposed to 1.50 mg of nicotine tartrate, autoresuscitation failure followed atrioventricular dissociation after cardiac resuscitation, as evidenced by an initial return of heart rate toward control levels; atrioventricular dissociation and ultimate loss of ventricular depolarization preceded the cessation of gasping (Fig. 6). As the level of nicotine exposure increased, however, gasping ceased before signs of cardiac resuscitation appeared on the electrocardiogram in a larger proportion of the pups (Fig. 7). Gasping ceased before signs of cardiac resuscitation appeared on the electrocardiogram in four of seven pups that were exposed to 3.00 mg of nicotine tartrate and in five of six pups that were exposed to 6.00 mg of nicotine tartrate. Perinatal exposure to nicotine did not alter the pattern of arousal or associated locomotor activity during an anoxic gas challenge.

Table 1. Sequence of events leading to autoresuscitation failure during repeat exposure to hypoxia following perinatal exposure to nicotine

<table>
<thead>
<tr>
<th>Nicotine Dose, mg·kg⁻¹·day⁻¹</th>
<th>A-V Dissociation After Cardiac Resuscitation</th>
<th>Cessation of Gasping Before Cardiac Resuscitation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6 of 7 Pups</td>
<td>1 of 7 Pups</td>
</tr>
<tr>
<td>1.50</td>
<td>5 of 7 Pups</td>
<td>2 of 7 Pups</td>
</tr>
<tr>
<td>3.00</td>
<td>3 of 7 Pups</td>
<td>4 of 7 Pups</td>
</tr>
<tr>
<td>6.00</td>
<td>1 of 6 Pups</td>
<td>5 of 6 Pups</td>
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A-V, atrioventricular.
DISCUSSION

Our experiments provide new information regarding perinatal exposure to nicotine and its ability to influence protective responses of newborn mammals to intermittent hypoxia. A novel finding in our study was that, although perinatal exposure to nicotine did not alter the time to last gasp or the total number of gasps during a single hypoxic exposure, it did impair the ability of rat pups to autoresuscitate from intermittent hypoxic-induced primary apnea in a dose-dependent manner. Maternal serum nicotine concentrations on the order of 19 ng/ml and greater resulted in premature failure of this important protective mechanism that promotes survival during intermittent hypoxia, as may occur in human infants during episodes of prolonged sleep apnea or positional asphyxia.

Nicotine is a neuroteratogen that easily crosses the placenta and is found in placental tissue, amniotic fluid, and fetal cord blood in concentrations equal to or greater than those measured in maternal blood (27, 28, 54). In the present experiments, fetal serum nicotine concentrations ranged from 91 to 240% of maternal serum nicotine concentrations on day 20 of gestation. Nicotine easily crosses the blood-brain-barrier (41), and prenatal exposure to nicotine has been shown to alter development of brain noradrenergic (35), cholinergic (50), dopaminergic (35), serotonergic (32), and vasopressinergic (59) systems in rodents; many of these effects persist into adulthood. Our present experiments establish for the first time the threshold level of maternal nicotine that significantly impairs protective responses of 5- to 6-day-old rat pups to repeat bouts of hypoxia, such as may occur in human infants during episodes of prolonged sleep apnea or positional asphyxia. This level of nicotine is similar to that observed in pregnant women who smoked an average of nine cigarettes over an 8-h period (i.e., 20 ± 8 ng/ml) or who wore a 21-mg nicotine patch over an 8-h period (i.e., 16 ± 4 ng/ml) in the study of Oncken et al. (36). Moreover, this level of nicotine is well below the plasma nicotine levels observed in moderate to heavy smokers (men and nonpregnant women) in the earlier
studies of Isaac and Rand (23) and Benowitz et al. (4). Although we would not argue with the proposition that cigarette smoking is more harmful than nicotine replacement therapy during pregnancy (3), our experiments provide evidence that nicotine alone and in concentrations similar to those achieved with the use of a nicotine patch can alter the physiology of the newborn such that they are more vulnerable to intermittent hypoxia from whatever cause.

In human infants, spontaneous recovery from obstructive sleep apnea or positional asphyxia during sleep is thought to occur early as a result of arousal from sleep or later as a result of hypoxic gasping, when it is known as “autoresuscitation” (17, 55). The arousal response from sleep, once characterized as “the forgotten response to respiratory stimuli” (39) is important for at least two reasons. First, wakefulness per se is a potent stimulus for maintenance of upper airway patency and to breathing, which are particularly important for resolution of obstructive apnea due to loss of upper airway muscle tone during sleep (42, 44). Second, arousal permits the initiation of an appropriate behavioral response, such as head turning, which is particularly important for resolution of positional asphyxia or obstructive apnea secondary to a face-straight-down sleeping position. Several groups of investigators have provided evidence that some infants at risk for SIDS, including those whose mothers smoked cigarettes during pregnancy, have a delayed arousal response to respiratory stimuli (26). If arousal and resumption of ventilation do not occur during obstructive sleep apnea or positional asphyxia before the partial pressure of oxygen in the arterial blood decreases to \(~10\) Torr, hypoxic cerebral depression and concomitant loss of electrocortical activity will occur (10, 25). The neurological status of the near-miss SIDS infant, when first discovered, has been termed “asphyxial coma,” resulting from hypoxic cerebral depression (56). During experimental asphyxial coma, whether produced by airway obstruction or by breathing an anoxic gas mixture (18, 25), the heart rate is decreased, electrocortical activity is absent, and eupneic breathing is replaced by prolonged apnea that is interrupted by occasional gasps. Experiments by Guntheroth et al. (18) and by Lawson and Thach (25) have shown that hypoxic-induced primary apnea and the onset of gasping occur when the partial pressure of oxygen in the arterial blood decreases to \(~8–10\) Torr;
this is true during hypercapnic hypoxia, as occurs during airway obstruction or during hypocapnic hypoxia as occurs during inhalation of a hypoxic gas mixture. With regard to survival, the crucial factor, and the focus of our present study, is whether gasping can produce autoresuscitation during asphyxial coma before the onset of terminal apnea and/or circulatory failure.

In our present experiments, exposure to hypoxia during a single anoxic gas challenge resulted in a reproducible respiratory response that consisted of hypopnea, primary apnea, gasping, and terminal apnea. Perinatal exposure to nicotine did not alter the time to last gasp or the total number of gasps (Fig. 3). This is similar to our previous results (11) as well as those of Schuen et al. (46), who found that perinatal exposure to 12 mg of nicotine tartrate/kg maternal body wt throughout gestation, which resulted in average maternal plasma nicotine concentrations of 134 ± 42 ng/ml, did not alter the time to last gasp on exposure to a single anoxic gas challenge in 6-day-old rat pups. Furthermore, Slotkin et al. (49) have shown that administration of ~6 mg of nicotine bitartrate (or ~2 mg of free nicotine base) per kilogram of maternal body weight throughout gestation does not increase the mortality rate of 1-day-old rat pups compared with control animals when they were exposed to 5% oxygen in nitrogen for 60 or 75 min. One- and four-day-old pups whose dam had received ~18 mg of nicotine bitartrate (or ~6 mg of free nicotine base) per kilogram of maternal body weight throughout gestation, however, had mortality rates nearly triple those of control animals during exposure to 5% oxygen in nitrogen for 75 min. These results suggest that perinatal exposure to nicotine would not alter the ability of an infant to respond to a single period of hypoxia as may occur during an initial episode of prolonged sleep apnea or positional asphyxia unless maternal nicotine levels were very high throughout gestation (i.e., perhaps >150 ng/ml; Refs. 49, 58). As previously reported by Gozal et al. (16), Fewell and Smith (11), Serdarevich and Fewell (47), and Fewell et al. (12), gasping occurred in three phases: an initial phase of rapid gasping (phase I), followed by a period of slower gasping (phase II), and finally a period of rapid gasping (phase III) that eventually waned and gave way to terminal apnea and death. Perinatal exposure to nicotine did not alter this gasping pattern.

The importance of gasping in self-resuscitation or autoresuscitation in infants has been emphasized by Peiper (38), Stevens (53), and Thach (55), as well as that repeat exposure to hypoxia can lead to autoresuscitation failure and death. Why autoresuscitation failure occurs is unclear, but clinical reports provide evidence that autoresuscitation can fail following repeat apneic episodes (38, 40, 51, 53). Successful autoresuscitation from hypoxic-induced apnea occurs in three sequential stages: stage I, gasping with marked bradycardia; stage II, cardiac resuscitation with a rapid increase in heart rate; and stage III, respiratory resuscitation with an increase in respiratory rate (11, 14, 47). Gershon et al. (15) have suggested that the three stages of autoresuscitation are accompanied by the following physiological events: first, introduction of air into the lungs by gasping; second, transport of oxygen from the lung to the heart; third, response of the heart by increasing heart rate and cardiac output; and, fourth, response of the central nervous system to reoxygenation and increased perfusion. Interestingly, Poets et al. (40) and Sridhar et al. (51) have recently provided evidence that some SIDS infants display stage I of autoresuscitation but that gasping fails to produce cardiac resuscitation (i.e., stage II of autoresuscitation) with resulting death.

In the present experiments, we found marked changes in the ability of rat pups to autoresuscitate during intermittent hypoxia when they were exposed to either 3.00 or 6.00 mg of nicotine tartrate·kg body wt⁻¹·day⁻¹ from day 6 or 7 of gestation. Furthermore, the sequence of events leading to autoresuscitation failure was influenced by nicotine dose. Previous experiments by St. John and Leiter (52) did not find that autoresuscitation from hypoxic-induced primary apnea was altered following prenatal exposure to nicotine. In their experiments, however, the rat pups underwent a single hypoxic challenge.

In the present experiments, most pups that received vehicle or 1.50 mg of nicotine tartrate during the perinatal period underwent stages I and II of autoresuscitation before the onset of atrioventricular dissociation, the loss of ventricular depolarization, and ultimate death; gasping continued throughout. This would allow one to suggest that cardiac output was maintained during hypoxia and that gasping resulted in the transport of oxygen from the lungs to the heart, resulting in reoxygenation and increased perfusion. Interestingly, the subsequent arrhythmia may have resulted from the accumulation of adenosine, which is a metabolic by-product of hypoxia that affects atrioventricular conduction (2, 5). In the majority of pups that were exposed to 3.00 or 6.00 mg of nicotine tartrate during the perinatal period, only stage I of autoresuscitation occurred before autoresuscitation failure. This would allow one to suggest that cardiac output was not maintained during hypoxia and that gasping did not result in the transport of oxygen from the lungs to the heart. The inability to maintain cardiac output during hypoxia may have resulted from nicotine-induced impairment of “nonneurogenic”-mediated release of catecholamines from the adrenal medulla (49) or perhaps from nicotine-induced depletion of the cardiac metabolic substrate glycogen, the cardiac stores of which are greater in the newborn than in older animals, which is important for maintenance of cardiac function during hypoxia (7, 48). Alternatively, it is possible that nicotine induced changes in one or all of the aforementioned brain neurotransmitter systems (e.g., for review, see Ref. 49) and subsequently altered the firing pattern of neurons located in the lateral tegmental field, which are essential for gasping in the rat (13), resulting in premature termination of gasping before cardiac resuscitation occurred. These mechanisms of autoresuscita-
tion failure after perinatal nicotine exposure are speculative and warrant further investigation. Regardless of the mechanism of autoresuscitation failure after perinatal exposure to nicotine, our data provide new insight into how maternal smoking may place an infant at risk for SIDS.

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