Respiratory syncytial virus infection in anesthetized weanling rather than adult rats prolongs the apneic responses to right atrial injection of capsaicin

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Peng W, Zhuang J, Harrod KS, Xu F. Respiratory syncytial virus infection in anesthetized weanling rather than adult rats prolongs the apneic responses to right atrial injection of capsaicin. J Appl Physiol 102: 2201–2206, 2007. First published March 15, 2007; doi:10.1152/japplphysiol.01436.2006.—Apnea is a common complication in infants infected by respiratory syncytial virus (RSV). A recent study has shown that intranasal inoculation of RSV in conscious weanling rats strengthens the apneic responses to right atrial injection of capsaicin (CAP), leading to 66% mortality. The objectives of the present study were to determine 1) whether RSV infection changes baseline minute ventilation (VE) and arterial blood gases in anesthetized rats; 2) what the effects of RSV infection are on the respiratory responses to CAP; and 3) whether the RSV-strengthened apneic responses are age dependent. Our experiments were conducted in anesthetized and spontaneously breathing rats divided into four groups of weanling and adult rats that received either intranasal inoculation of RSV or virus-free medium. Two days after RSV infection (0.7 ml/kg), animal blood gases, baseline VE, and VE responses to right atrial injection of three doses of CAP (4, 16, and 64 μg/kg) were measured and compared among the four groups. Our results showed that RSV infection increased respiratory frequency (~25%, P < 0.05) in weanling but not adult rats, with little effect on arterial blood gases. RSV infection amplified the apneic responses to CAP in weanling but not adult rats, characterized by increases in the initial (40%) and the longest apneic duration (650%), the number of apneic episodes (139%), and the total duration of apneas (60%). These amplifications led to 50% mortality (P < 0.05). We conclude that RSV infection increases respiratory frequency and strengthens the apneic responses to CAP only in anesthetized weanling but not adult rats.

METHODS

All procedures were conducted under protocols approved by the Institutional Animal Care and Use Committee in Lovelace Respiratory Research Institute facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Virus preparation and inoculation. A total of 29 weanling (2 wk) and 23 adult (8 wk) male F344 rats were used. The pathogen-free rats were obtained from Taconic Laboratories (Germantown, NY). The average body weight of the weanlings and adults at the time of the experiment were 30 ± 4 g and 203 ± 6 g, respectively. RSV (A2 strain) was propagated in Hep-2 cell cultures, as described previously (11). Briefly, Hep-2 cells were grown in Eagle’s minimum essential medium supplemented with 10% FBS (GIBCO-BRL, Grand Island, NY). Confluent monolayers of Hep-2 cells were infected with 0.1 plaque-forming units (pfu) of human RSV (A2 strain), and the infection was incubated at 37°C in a 5% CO2 atmosphere until ~75% of the cells exhibited cytopathic effect. Purified RSV titers were determined in duplicate for each culture dilution using standard plaque assay procedures. Plaque formation was counted manually by visualization following neutral red-stained monolayers (11, 22). Aliquots of the virus stock were snap-frozen and stored in liquid nitrogen. Before inoculation, the viral stock was titrated and diluted to the final titer of 3.5 × 106 pfu/ml. It should be noted that the same RSV strain, concentration, instillation approach, and duration previously used in Fisher rats have been shown to cause a RSV infection limited to the upper airway infection; bronchopulmonary C fibers

Respiratory syncytial virus (RSV) infections result in hospitalization of ~120,000 children annually in the United States (9, 28), and apnea may be the first sign of the disease (15, 27, 29). A recent study showed that RSV infection in weanling rats significantly prolonged apneic duration in response to right atrial injection of capsaicin (CAP; >5.0 μg/kg), leading to 66% mortality (26). Interestingly, hypoxemia can also prolong the CAP-induced apnea (36). Right atrial injection of CAP causes a brief apnea (4 s), whereas the same CAP injection during acute hypoxia (10% O2 for 1 min) generates a long-lasting apnea (69 s) in anesthetized rats. It thus becomes necessary to clarify whether RSV infection in weanling rats produces hypoxemia that partially contributes to the prolongation of the apneic response to CAP. A large dose of right atrial bolus injection of CAP (>5.0 μg/kg) could lead to several apneic episodes (26), in which the immediate apnea is mediated by stimulating bronchopulmonary C fibers (PCFs) (4–6, 8, 13, 20, 25, 35, 38). The following apneas may be involved in the more complicated mechanisms. Defining to what degree RSV prolongs the initial and/or the following apneas is helpful in further investigating the mechanisms of RSV amplification of the CAP-induced apnea. In addition, apnea and respiratory failure secondary to acute respiratory disease often occur in younger infants (1, 3, 15, 24), and the morbidity of RSV infection is closely related to age (15, 27, 29). This higher apneic susceptibility in earlier life seems to be related to a supersensitivity of the PCF-mediated respiratory response. A stronger apneic response to CAP has been observed in postnatal rats compared with that of adult rats (33). Therefore, it is important to delineate whether the strengthened apneic responses observed in RSV-infected weanling rats is age dependent. In the present study, we hypothesized that 1) compared with the control rats, hypoxemia occurs in anesthetized weanling rats 2 days after RSV intranasal inoculation; 2) this RSV infection prolongs both the initial and subsequent apneic durations in response to right atrial injection of CAP; and 3) these RSV-induced respiratory responses are greater in the weanling than adult rats.

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upper respiratory tract (26). Our experiments were conducted in anesthetized spontaneously breathing rats divided into four groups: the weanling and adult rats received either RSV intranasal inoculation (W-RSV and A-RSV) or virus-free medium (W-CON and A-CON). The W-RSV and A-RSV rats were inoculated intranasally under isoflurane anesthesia by depositing the virus suspension (0.7 ml/kg) into each nostril. The same volume of pathogen-free medium (RPML-1640) served as vehicle control for the W-CON and A-CON rats. Following inoculation, the rats were housed under pathogen-free conditions in a designated Animal Biosafety Level-2 facility for 2 days. The infected and pathogen-free rats were placed in separate rooms and housed under strict barrier and pathogen-free conditions according to Association for Assessment and Accreditation of Laboratory Animal Care International-approved guidelines and protocols to prevent any microbial contamination. On day 3, the rats were placed inside a flow hood, and their baseline ventilation, arterial blood gases, and ventilatory responses to right atrial injection of CAP were measured as described below.

**Arterial blood sampling.** Twenty-four rats (n = 6 for each group) were anesthetized with pentobarbital sodium (Nembutal, 50 mg/kg intraperitoneally) to suppress corneal and withdrawal reflexes. The right femoral artery was isolated and cannulated, and 135 μl of arterial blood were collected in a heparinized capillary tube and analyzed immediately by using the GEM Premier 3000 blood gas analyzer (Instrumentation Laboratory, Lexington, MA).

**Measurement of baseline minute ventilation and ventilatory responses to CAP.** After anesthetization as described above, the right jugular vein was isolated and a catheter advanced close to the right atrium for injection of CAP in each of the 28 other rats (n = 7, 10, 5, and 6 for W-CON, W-RSV, A-CON, and A-RSV, respectively). The inserted depth of the catheter was determined by measuring the distance from the heart (felt from the heartbeat) to the cannulation site before implantation. The position of the cannula was confirmed by autopsy after the experiments. PE-50 tubing was used in the adult rats, and the tip of the tubing was narrowed by heat-pulling to allow for insertion in weanling animals. Immediately before animals were placed into the plethysmograph, the anesthetic level was checked, and if necessary supplemental anesthetic added. When the animal was placed in a whole body plethysmograph (unrestrained) (Buxco Electronic, Sharon, CT) to measure minute ventilation (Ve), the tubing of the jugular cannulation and the wire of thermo-probe for measuring rectal temperature exited through an outlet of the plethysmograph that was then sealed. If a sudden increase in respiratory rate and/or ventilation (>20%) under control was observed and these changes kept increasing continuously within 10 min, appropriate supplemental anesthetic was administered via jugular cannulation. The plethysmograph was placed on a heating pad, and animal rectal temperature was maintained at 36.5 ± 0.6°C with no difference between the four groups of rats (P > 0.05). After stabilization of respiratory activity for 5 min, the animal received right atrial injection of three different doses of CAP (Sigma-Aldrich, St. Louis, MO), i.e., low dose (4 μg/kg), medium dose (16 μg/kg), and high dose (64 μg/kg). At a given CAP dose, the concentration was the same in weanling and adult rats, whereas the volume to be injected was adjusted according to the doses as previously reported (33). CAP was dissolved in vehicle solution containing 10% Tween-80, 10% ethanol, and 80% isonicotinic saline. A 15-min interval was allowed between two stimulations. Routinely, equivalent volumes of vehicle were injected in control animals to serve as the sham control.

**Data acquisition and analysis.** Airflow signals from the Buxco system were used to calculate Ve, tidal volume (VT), and respiratory frequency (f). These signals were also simultaneously monitored and recorded by a PowerLab/8sp (ADInstruments, Colorado Springs, CO) connected to a computer employing the PowerLab Chart 5 software. An apneic response was defined as a threefold prolongation of baseline expiratory time after each CAP administration. In the present study, the severity of apneic responses was characterized by four variables: 1) the initial apnea occurring immediately after CAP injection; 2) the longest apnea; 3) the number of apneic episodes; and 4) total duration of apneas (sum of apneic durations induced by CAP). The initial apnea was fully mediated by stimulation of PCFs, whereas the longest apnea likely reflected CAP action on PCFs and the central nervous system. The number of apneic episodes and total duration of apneas were used to determine the severity of the apneic responses. In addition, the latencies of the initial apnea were defined as the duration between the onsets of CAP injection and the immediate apnea. All values were presented as means ± SE. The differences of baseline values of ventilatory activity and arterial blood gas analysis among animal groups were analyzed by two-way ANOVA. The ventilatory differences by the low, medium, and high dose of CAP injection between the weanling and adult rats treated with RSV or virus-free medium were tested by using two-way ANOVA with repeated measures. When the interaction term of the ANOVA test was found to be significant, Fishers least significant difference was followed for multiple comparisons. P values of <0.05 were considered significant.

**RESULTS**

**Baseline Ve and arterial blood gases.** We compared arterial blood gases in the four groups of rats. As shown in Table 1, arterial blood gases (arterial PO2, PCO2, pH, and O2 saturation) were not significantly different between the two age groups and between the rats with and without RSV infection. VT and Ve were markedly higher in the A-CON rats than in the W-CON rats with no remarkable difference in f. Faster breathing (~25% increase in f) was observed in W-RSV rats compared with W-CON (P < 0.05) rats without significant changes in VT and Ve. In comparison, the respiratory variables were not different between A-CON and A-RSV rats. The typical recordings of baseline respiratory variables in four groups of rats are illustrated in Fig. 1. A and B, respectively, whereas their corresponding group data are presented in Fig. 1C.

**Ventilatory responses to CAP.** Low-dose CAP delivered into the right atrium evoked a single apnea in both W-CON and A-CON rats, but multiple apneas in all W-RSV and in 50% of A-RSV rats were observed. Fifty percent of the remaining A-RSV rats only displayed a single apneic event. In contrast, medium- and high-dose CAP generated multiple apneas in all animals and both age groups with or without RSV infection. Figure 2 displays the typical experimental recordings showing the ventilatory responses to three doses of CAP in the W-RSV group. Multiple apneas were typically composed of an initial apnea immediately after CAP injection followed by several subsequent apneas. The longest apnea usually appeared after several shorter apneas (Fig. 2, top and middle). In all groups of animals, vehicle injection did not significantly change expiratory time (0.34 ± 0.02 vs. 0.42 ± 0.06 s; P > 0.05).

<table>
<thead>
<tr>
<th>Groups</th>
<th>pH</th>
<th>Paco2 Torr</th>
<th>Paco2 Torr</th>
<th>Sao2 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>W-CON</td>
<td>7.34 ± 0.04</td>
<td>42.0 ± 3.4</td>
<td>80.3 ± 6.3</td>
<td>90.3 ± 6.0</td>
</tr>
<tr>
<td>W-RSV</td>
<td>7.31 ± 0.04</td>
<td>38.9 ± 4.6</td>
<td>75.1 ± 9.2</td>
<td>87.2 ± 5.6</td>
</tr>
<tr>
<td>A-CON</td>
<td>7.30 ± 0.02</td>
<td>43.2 ± 2.1</td>
<td>86.6 ± 8.3</td>
<td>93.5 ± 4.3</td>
</tr>
<tr>
<td>A-RSV</td>
<td>7.28 ± 0.06</td>
<td>42.5 ± 3.6</td>
<td>84.7 ± 10.5</td>
<td>91.8 ± 5.9</td>
</tr>
</tbody>
</table>

The variables are means ± SE (n = 6 in each group). W-CON, weanling virus-free; A-CON, adult virus-free; W-RSV, weanling RSV infected; A-RSV, adult RSV infected; Paco2, arterial PCO2; Paco2, arterial PO2; Sao2, arterial O2 saturation.

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Numbers of apneic episodes and total durations of apneas.

Right atrial injection of CAP significantly increased the number of apneic episodes and the total duration of apneas in a CAP dose-dependent manner, showing a dependency of these apneic responses on PCF stimulating intensity. In control rats, the number of apneic episodes was not significantly different between the two age groups (Fig. 3). However, RSV infection significantly increased the number of apneic episodes in response to medium- and high-dose CAP in weanling but not in adult rats. We also compared the total duration of apneas (the sum of apneic durations) among the four groups for all three CAP doses tested (Fig. 4). It was not significantly different between W-CON and A-CON rats. Interestingly, apnea was significantly prolonged by RSV in the weanling but not adult rats.

The initial and longest apneic durations. Bolus injection of CAP caused an immediate apnea. The latencies of the apnea evoked by low-, medium-, and high-dose CAP were 1.7 ± 0.1, 1.6 ± 0.1, and 1.7 ± 0.2 s for the control, and 1.8 ± 0.2, 1.6 ± 0.2, and 1.6 ± 0.2 s for the RSV-infected weanling rats. In adult rats, the latencies evoked by the three doses were 1.7 ± 0.1, 1.7 ± 0.1, and 1.5 ± 0.1 s for the control, and 1.7 ± 0.1,
1.6 ± 0.2, and 1.7 ± 0.1 s for the RSV-infected rats. The latencies were not significantly affected by CAP doses, age, or RSV infection. The initial apneic durations in response to the three doses of CAP were not different between the W-CON and A-CON rats. But RSV infection significantly increased the responses of the initial apneic duration to CAP only in the weanling rats (Fig. 5). The longest apneic duration usually appeared 10 s after CAP injection. The CAP response of the longest apneic duration (Fig. 6) was significantly different between the weanling and adult rats. RSV failed to alter these variables in the adult rats; however, it did significantly enhance them in weanling rats. It should be noted that, in weanling rats, the RSV-induced changes in the longest apneic duration were much greater compared with the initial apneic duration.

Mortality induced by right atrial injection of CAP in RSV-infected anesthetized weanling rats. CAP failed to generate any mortality in control rats; however, RSV treatment did cause 50% mortality uniquely in weanling rats (5/10), in which the “final apnea” often appeared several seconds following the longest apnea and led to death. Of these five weanling rats, four died when a high dose of CAP was administered, and one died even when a low dose was given. In addition, compared with W-CON rats, the amplitude of the RSV infection-induced changes of the apneic responses to CAP in W-RSV rats was ranked as the longest apneic duration (650%) > the apneic episodes (139%) > the total duration of apneas (60%) > the initial apneic duration (40%).

DISCUSSION

Previous studies have shown the RSV infection-induced tachypnea in postnatal lambs (23) and human infants (2). In agreement, tachypnea was also observed in our weanling rats with no changes in arterial blood gases. Since RSV infection is often associated with fever, it is assumed that the tachypnea might result from the RSV-induced fever (7, 10). Our data do not support this assumption because animal body temperature was maintained at a similar level in control and RSV-treated anesthetized rats. The mechanisms of RSV-induced tachypnea remain unknown. The absence of a lower respiratory tract infection, as reported previously (26), may account for the lack of discernible changes in arterial blood gases in the RSV-infected rats in our study.

There are several lines of evidence in the study leading to the conclusion that RSV infection amplifies the CAP-induced apneic responses in weanling rather than adult rats. First, multiple apneas were observed in response to a low dose of CAP in all RSV-infected weanlings, but only 50% of adult rats exhibited this response. Second, RSV infection significantly increased all of the four apneic parameters in the weanling but not adult rats. Third, 50% mortality was found in RSV-infected weanlings when CAP was administered; however, no mortality occurred in adult rats. The supersensitivity of PCFs in early life may be partially responsible for the RSV infection amplification of the apneic responses observed in weanling rats. Morphologically, the number of vagal C fibers was reported to be higher in preterm lambs (12, 24). Functionally, a stronger apneic response to right atrial injection of CAP was observed in earlier life of anesthetized rats (33). On the other hand, we cannot rule out other possibilities. For example, compared with the adults, the weaker immune reactivity and greater physical fragility in earlier life (1, 3, 9, 15, 24, 27–29) may partially contribute to the stronger apneic responses to CAP.

We found that the latency to evoke the initial apnea was ~1.7 s in weanling and adult rats, similar to that previously reported in postnatal and adult anesthetized rats and cats (20, 33, 34, 36, 37). Interestingly, in the present study, RSV infection prolonged the initial apneic duration by 40%, which is much smaller than the CAP impacts on the following apneas (the longest apneic duration was prolonged by 650% and the apneic episodes by 139%). The immediate apneic response to CAP is mediated fully by stimulating PCFs (4, 5, 14, 17–19, 25, 34). A large body of previous studies has demonstrated that right atrial bolus injection of CAP (≤5 μg/kg) produces an apnea that is completely eliminated by bivagotomy (4, 5, 14, 17–19, 25, 34), supporting the predominant role of PCFs in the apneic genesis. It remains unclear whether PCFs are also fully responsible for the following apneas (death) as observed in our W-RSV rats when a high does of CAP (64 μg/kg) was injected.

There are some limitations in our experiment. In conscious RSV-infected weanling rats, 66% mortality occurred after right
atrial injection of 10 μg/kg CAP (26). In our experiment, mortality (50%) in anesthetized weanling rats is produced by injection of a much greater dose of CAP (64 μg/kg). In accordance with this finding, the total duration of apneas in response to CAP was relatively shorter in our anesthetized weanling rats compared with conscious weanling rats previously reported (26). These results suggest that RSV amplification of PCF-mediated apneic response exists in anesthetized weanling rats, although anesthesia has an inhibitory effect on the CAP-induced apneic responses. We recognized that it would be optimal if the cardiopulmonary (arterial blood gases) variables and their responses to CAP could be measured in conscious animals. However, our pilot experiments showed that the vessel wall of the femoral artery in F344 weanling rats was too small and fragile to chronically implant a catheter for recording heart rate and arterial blood pressure. Because of a lack of cardiovascular data in the present study, we cannot delineate the contributions of respiratory and/or cardiac failure to the mortality mentioned above. It has been well documented that RSV infection is the major cause of childhood bronchiolitis (9, 28). Therefore, we measured ventilation in a plethysmograph instead of using a pneumotachograph to prevent tracheal cannulation-induced interference with measurements of ventilatory function.

In summary, our results show that acute RSV infection does not alter arterial blood gases but significantly affects respiration in weanling rather than adult rats. These functional changes are characterized by tachypnea and augmentation of the apneic responses to right atrial injection of CAP. These results suggest that acute RSV infection in the upper respiratory tract leads to heightened apneic responses in anesthetized weanling but not adult rats and that PCFs are involved in the RSV-induced apnea.

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REFERENCES


