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Ventilatory function assessment in safety pharmacology: Optimization of rodent studies using normocapnic or hypercapnic conditions

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ABSTRACT

Although the whole body plethysmography for unrestrained animals is the most widely used method to assess the respiratory risk of new drugs in safety pharmacology, non-appropriate experimental conditions may mask deleterious side effects of some substances. If stimulant or bronchodilatory effects can be easily evidenced in rodents under standard experimental conditions, i.e. normal air breathing and diurnal phase, drug-induced respiratory depression remains more difficult to detect. This study was aimed at comparing the responsiveness of Wistar rats, Duncan Hartley guinea-pigs or BALB/c mice to the respiratory properties of theophylline (50 or 100 mg/kg p.o.) or morphine (30 mg/kg i.p.) under varying conditions (100% air versus 5% CO_2-enriched air, light versus dark day phase), in order to select the most appropriate experimental conditions to each species for safety airway investigations. Our results showed that under normocapnia the ventilatory depressant effects of morphine can be easily evidenced in mice, slightly observed in guinea-pigs and not detected in rats in any day phase. Slight hypercapnic conditions enhanced the responsiveness of rats to morphine but not that of guinea-pigs and importantly they did not blunt the airway responsiveness of rats to the stimulation and bronchodilation evoked by theophylline, the most widely used reference agent in safety pharmacology studies. In conclusion, hypercapnic conditions associated with the non-invasive whole body plethysmography should be considered for optimizing the assessment of both the ventilatory depressant potential of morphine-like substances or the respiratory stimulant effects of new drugs in the rat, the most extensively used species in rodent safety and toxicological investigations.

Introduction

Barometric whole body plethysmography for unrestrained animals is a widely used method to assess the ventilatory function with numerous advantages (Al Qadi-Nassar et al., 2007; Chong et al., 1998; Hamelmann et al., 1997; Strohl et al., 1997). Compared with the double chamber plethysmography, it avoids handling-induced animal stress and allows continuous monitoring of the respiratory cycle for relatively long periods i.e. up to several hours. Compared with anesthetized animal models, it avoids any pharmacological interference with anaesthetic agents and enables using the same animals for repeated measurements. Thus, this technique is extensively employed for safety evaluation of potential ventilatory side effects of new drugs (Murphy, 2003) or for various efficacy studies on respiratory disorders such as asthma or chronic obstructive pulmonary disease (Duan et al., 2003; Lin, 2001; Ram et al., 2008; Sharma et al., 2009; Spond et al., 2004). Nevertheless, these experiments require that the conscious animals have to be appropriately acclimated to the experimental chamber to ensure stabilized breathing pattern (Bazan-Perkins et al., 2004).

Mainly in rodents, this non-invasive method is recognized as being relevant to detect the ventilatory stimulant properties of drugs which increase the respiratory rate and/or the minute ventilation (De Sanctis et al., 1991) or the allergen specific or non-allergic respiratory responses under pathophysiological conditions in rats (Spond et al., 2004), mice (Al Qadi-Nassar et al., 2007; Lin, 2001) or guinea-pigs (Chavez et al., 2007). However, plethysmographic measurements in rodents could be less sensitive for detecting ventilatory depressant effects under standard conditions (normal air, light cycle, quiet environment, undisturbed animals, etc.). Therefore, the experimental conditions should be adapted in order to insure that the rodent models are sensitive enough as well to the potential ventilatory depressant side effects as to the potential respiratory stimulant properties of new drugs, especially when using rats which remain the species the most extensively used in rodent safety and toxicological investigations (Chevillard et al., 2009; Montandon et al., 2006; Villa et al., 2007).

The rodent, which is nocturnal, is predominantly awake and active during the night whereas it is mainly resting or asleep during the day (Trachsel et al., 1986). The normal breathing pattern could be therefore stimulated during the dark cycle. In addition, the exposure

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to CO_2 gas is known to stimulate ventilation and the recording of the respiratory cycle under hypercapnic conditions could be another alternative for enhancing the likelihood to detect respiratory depressant effects (Nettleton et al., 2007; Van den Hoogen and Colpaert, 1986).

The present study was designed to compare the responsiveness of the three main rodent species used in safety pharmacology, i.e. rat, guinea-pig and mouse to the ventilatory depressant effects of morphine administered at the same dose in all species, under normal or modified experimental conditions (dark cycle on rats or hypercapnic conditions on the three rodent species). In addition, for the species showing an increased responsiveness to morphine-induced ventilatory depression under specific conditions, we verified whether these adapted experimental conditions do not impair the sensitivity of the model to detect the ventilatory stimulant effects of theophylline, the most widely used reference substance in safety investigations.

Methods

Animals. Male Rj: Wistar (Han) rats (weighing 400 ± 50 g), male Rj: BALB/cJ mice (weighing 25 ± 5 g) and male Duncan Hartley guinea-pigs (weighing 350 ± 80 g) were used. Rats or mice were supplied by Elevage Janvier, 53940 Le Genest-Saint-Isle, France and guinea-pigs were supplied by Charles River Laboratories, 69592 L'Arbresle, France.

Animals were delivered to the laboratory at least 5 days before the experiment, during which time they were acclimatized to the laboratory conditions. Rats, guinea pigs or mice were housed in groups of 2 to 4 in macrolon cages with free access to food and water until tested. The animal house was maintained under artificial lighting (12 h) between 7:00 a.m. and 7:00 p.m. at a controlled ambient temperature of 21 ± 3 °C and relative humidity of 30–80%.

At the end of the experiments, the animals were terminated by exposure to O_2/CO_2 (20%/80%) followed by 100% CO_2.

All experiments were performed in accordance with French legislation concerning the protection of laboratory animals and in accordance with a currently valid license for experiments on vertebrate animals issued by the French Ministry for Agriculture and Fisheries.

Investigated variables. Animals, not deprived of food, were placed individually in a whole barometric body single chamber plethysmograph (EMKA Technologies, France), ventilated by continuous bias flow of 2 L/min (EMKA Technologies), in which they can move and drink freely. A differential pressure transducer was connected to the chamber and pressure signals were amplified, digitized and sampled at 100 Hz using validated and specialized software (IOX version 1.7.0, EMKA Technologies, France). The following parameters derived from the box pressure changes during the respiratory cycle were analysed (ANALYST version 1.49, EMKA Technologies, France):

- Inspiratory time (Ti, ms), defined as the time from the start of inspiration to the end of inspiration;
- Expiratory time (Te, ms), defined as the time from the end of inspiration to the start of the next inspiration;
- Peak inspiratory flow (PiF, mL/s), defined as the maximum box pressure signal occurring during one breath in a negative direction;
- Peak expiratory flow (PeF, mL/s), defined as the maximum box pressure signal occurring during one breath in a positive direction;
- Tidal volume (TV, mL), defined as the integral of inspiratory (negative) time;
- Respiratory rate (ResR, breaths/min), extrapolated from recordings of every 10 breaths;
- Minute volume (minV, mL/min) = TV x ResR.

Experimental design. The animals were allowed to acclimatize to the whole body plethysmography chamber for a 150-min period under normal conditions (i.e. breathing room air) or hypercapnic conditions (i.e. exposition to 5% CO_2 in air). The animals received the pharmacological or vehicle treatment after the stabilization period and they were immediately reintroduced into the plethysmographic chamber.

Recordings were taken for 30 min before (baseline measurements used as T0 values) and for 2 h (intraperitoneal administration), 4 h (oral administration) or 12 h (dark cycle experiments) after administration of the test substance. Each recorded value was the mean value obtained from a block of 2 min recording (made 1 min before and 1 min after each time point).

The respiratory effects of theophylline (100 mg/kg p.o. for rats or guinea-pigs; 50 mg/kg p.o. for mice) and morphine (30 mg/kg i.p. for all species) were evaluated in the three rodent species under normocapnic conditions and compared with appropriate vehicle control groups (0.2% hydroxypropylmethylcellulose, HPMC, p.o. or physiological saline i.p.). The ventilatory effects of morphine were also evaluated under modified experimental conditions, i.e. during the dark cycle for the rat or under hypercapnic conditions (5% CO_2/95% air) for the three rodent species. Lastly, the effects of theophylline (100 mg/kg p.o.) were also assessed in the rat placed under hypercapnic conditions. Each treatment was evaluated in separate groups of naïve animals.

Drugs. Theophylline was obtained from Sigma-Aldrich (St Quentin, France) and morphine was obtained from Coopération Pharmaceutique Française (Melun, France).

The doses of theophylline and morphine were selected on the basis of preliminary internal data obtained on the general behavior of rats or mice using the primary behavioral observation (Irwin) test (unpublished data). The dose of 100 mg/kg p.o. of theophylline was chosen as a dose inducing significant stimulation on the respiratory function without observable deleterious lateral effects, except for BALB/cJ mice in which lethality was observed. In this species, the dose of theophylline was decreased to 50 mg/kg p.o. The dose of 30 mg/kg i.p. of morphine was retained as the dose susceptible to evoke a ventilatory depression, with a specific species-dependent responsiveness, without inducing a marked sedation in the animals kept freely moving in their plethysmography single chamber.

Statistical analysis. Reported values are expressed as means ± S.E.M. ($n = 8$ animals per group). Statistical analysis was performed using SAS® Version 8.2 or EXCEL Version 2003. Intra-group comparisons were performed for control groups using a one-way analysis of variance (time) with repeated measures at each time, followed by Dunnett's tests in case of significant time effect, to compare each time value with the T0 value (i.e. basal value before vehicle administration). Inter-group comparisons were performed using a two-way analysis of variance (group, time) with repeated measures at each time, followed by a one-way analysis of variance (group) at each time in case of significant group x time interaction. The analysis was completed by Dunnett's tests where group effect was significant.

The area under curve (AUC) was calculated by the trapezoidal rule. Inter-group comparison was performed using unpaired Student's *t* tests.

Results

Effects of theophylline under normal air conditions

Under normal air conditions, a transient increase in minute volume occurred shortly following the administration of vehicle in rats, guinea-pigs and mice with a maximum at time point T10 min post-administration (vehicle control groups). This effect is classically ascribed to the stress induced in the animals by handling during the