Toxicology 305 (2013) 99–108

Contents lists available at SciVerse ScienceDirect

Toxicology

journal homepage: www.elsevier.com/locate/toxicol

Gender and strain contributions to the variability of buprenorphine-related respiratory toxicity in mice

Hisham Alhaddad[a,b], Salvatore Cisternino[a,b], Bruno Saubamea[a,b], Joel Schlatter[c], Fouad Chiadmi[c], Patricia Risède[a,b], Maria Smirnova[a,b], Véronique Cochois-Guégan[a,b], Nicolas Tournier[d], Frédéric J. Baud[a,b,e,f], Bruno Mégarbane[a,b,e,f,*]

[a] INSERM U705, CNRS UMR8206, Neuropsychopharmacologie des addictions, Faculté des Sciences Pharmaceutiques et Biologiques, Paris, France
[b] Faculté de Pharmacie, Université Paris Descartes, Sorbonne Paris Cité, 75006 Paris, France
[c] Assistance Publique – Hôpitaux de Paris, Hôpital Jean Verdier, Laboratoire de Toxicologie, Bondy, France
[d] CEA, I2BM, Service Hospitalier Frédéric Joliot, Orsay, France
[e] Assistance Publique – Hôpitaux de Paris, Hôpital Lariboisière, Réanimation Médicale et Toxicologique, Paris, France
[f] Université Paris-Diderot, Sorbonne Paris Cité, F-75205 Paris, France

ARTICLE INFO

Article history:
Received 18 November 2012
Received in revised form 14 January 2013
Accepted 23 January 2013
Available online 29 January 2013

Keywords:
Buprenorphine
P-glycoprotein
Respiratory depression
Gender differences
Strain differences
Blood–brain barrier

ABSTRACT

While most deaths from asphyxia related to buprenorphine (BUP) overdose have been reported in males, higher plasma concentrations of BUP and its toxic metabolite norbuprenorphine (NBUP) have been observed in females. We previously demonstrated that P-glycoprotein (P-gp) modulation at the blood–brain barrier (BBB) contributes highly to BUP-related respiratory toxicity, by limiting NBUP entrance into the brain. In this work, we sought to investigate the role of P-gp-mediated transport at the BBB in gender and strain-related variability of BUP and NBUP-induced respiratory effects in mice. Ventilation was studied using plethysmography, P-gp expression using western blot, and transport at the BBB using *in situ* cerebral perfusion. In male Fvb and Swiss mice, BUP was responsible for ceiling respiratory effects. NBUP-related reduction in minute volume was dose-dependent but more marked in Fvb ($p < 0.01$ at 1 mg/kg NBUP and $p < 0.001$ at 3 and 9 mg/kg NBUP) than in Swiss mice ($p < 0.001$ at 9 mg/kg NBUP). Female Fvb mice were more susceptible to BUP than males with significantly increased inspiratory time ($p < 0.05$) and to NBUP with significantly increased expiratory time ($p < 0.01$). Following BUP administration, plasma BUP concentrations were significantly higher ($p < 0.01$) and plasma NBUP concentrations significantly lower ($p < 0.001$) in Fvb mice compared to Swiss mice. Plasma BUP concentrations were significantly higher ($p < 0.05$) and plasma NBUP concentrations significantly lower ($p < 0.01$) in male compared to female Fvb mice. In contrast, following NBUP administration, comparable plasma NBUP concentrations were observed in both genders and strains. No differences in P-gp expression or BUP and NBUP transport across the BBB were observed between male and female Fvb mice as well as between Swiss and Fvb mice. Our results suggest that P-gp-mediated transport across the BBB does not play a key-role in gender and strain-related variability in BUP and NBUP-induced respiratory toxicity in mice. Both gender- and strain-related differences in respiratory effects of BUP could be attributed to BUP itself rather than to its metabolite, NBUP.

1. Introduction

Buprenorphine (BUP) has been marketed as efficient maintenance therapy in heroin addicts (Gowing et al., 2009). Since its marketing, poisonings and fatalities from asphyxia have been reported (Mégarbane et al., 2010; Kintz, 2001). Respiratory toxicity was mainly attributed to BUP misuse and association with benzodiazepines (BZD). BUP is metabolized to norbuprenorphine (NBUP) by cytochrome P450 (CYP)-mediated N-dealkylation in the liver (Iribarne et al., 1997). NBUP is an active compound with potent respiratory depressant effects (Mégarbane et al., 2006; Ohtani et al., 1997); however, its exact role in BUP toxicity remains poorly understood. Recently, inhibition of P-glycoprotein (P-gp; mdr1a)-mediated efflux of NBUP at the blood–brain barrier (BBB) was shown to significantly enhance BUP-related respiratory effects (Alhaddad et al., 2012).

In humans, gender differences in opioid toxicity are underevaluated due to the higher rate of substance abuse in men than

* Corresponding author at: Réanimation Médicale et Toxicologique, Hôpital Lariboisière, 2 Rue Ambroise Paré, 75010 Paris, France. Tel.: +33 149958961; fax: +33 149956578.
E-mail address: bruno-megarbane@wanadoo.fr (B. Mégarbane).

0300-483X/$ – see front matter © 2013 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.tox.2013.01.013

women, although the gap has been closing in the recent years (Unger et al., 2010). Recently, gender-related differences have been demonstrated in BUP pharmacokinetics. Females have been shown to present significantly higher area under the curve (AUC) and peaks of plasma concentrations for BUP, NBUP, and NBUP-3-glucuronide in comparison to males (Moody et al., 2011). These differences were attributed to differences in body composition and CYP3A-dependent metabolism of BUP. However, although these differences may hypothetically make females more vulnerable to BUP toxicity, the majority of poisonings and fatalities are observed in males (Pirnay et al., 2004).

Animal investigations showed marked NBUP-related respiratory effects in both female Fvb (Alhaddad et al., 2012) and male CF-1 mice (Brown et al., 2012). In female P-gp knock-out Fvb mice, increased NBUP-related respiratory effects were reported (Alhaddad et al., 2012). Similarly, in male P-gp deficient CF-1, NBUP-mediated antinociceptive as well as respiratory effects were enhanced (Brown et al., 2012). However, to date, no data exist regarding differences in BUP and NBUP-related toxicity attributable to gender and strain in mice. Our objectives were to describe gender- and strain-related variability of BUP and NBUP-induced respiratory effects in mice and to investigate the exact contribution of P-gp-mediated transport of NBUP at the BBB in the observed differences.

2. Methods

All experiments were carried out within the ethical guidelines established by the National Institutes of Health and the French Ministry of Agriculture. All experimental protocols were approved by the IMTCE animal facility experimental procedures (Paris-Descartes University) and by our institutional ethic committee.

2.1. Animals

Male and female Fvb (20–25 g) and male Swiss mice (20–25 g) were purchased from Janvier (Genest, France). Animals were housed in well-ventilated cages at 20–22 °C with 55 ± 10% relative humidity and maintained under a 12-h dark/light cycle (light from 8:00 a.m. to 8 p.m.) for at least 1 week before the experiments. Food and water were provided *ad libitum*. Following each experiment, mice were euthanized using a carbon dioxide chamber.

2.2. Chemicals and drugs

BUP hydrochloride and NBUP were generously provided by Schering-Plough (Courbevoie, France). BUP (1 mg/ml) and NBUP (0.1 mg/ml) administration were diluted in 4% Tween® (Sigma, St. Quentin, France) for intraperitoneal (i.p.) use. [^{3}H]-BUP (80–85 Ci/mmol) was purchased from American Radiolabelled Chemicals (Saint Louis, MO, USA). [^{14}C]-Sucrose (588 mCi/mmol) was purchased from Perkin Elmer Life Sciences (Courtaboeuf, France). d4-BUP and d3-NBUP were purchased from Cerilliant (Molsheim, France). All other chemicals were of analytical grade and purchased from Sigma.

2.3. Whole body plethysmography

Ventilatory parameters were recorded in a whole body plethysmograph by the barometric method described and validated in the rat (Bartlett and Tenney, 1970). Mice were placed in a rectangular Plexiglas chamber and restrained for measurement of ventilatory parameters and rectal temperature using a Plexiglas cylinder (internal diameter: 2.5 cm, adjustable length up to 10 cm) (Harvard apparatus Inc., Holliston, MA). This chamber was connected to a reference chamber of the same size by a high resistance leak to minimize the effect of pressure changes in the experimental room. The animal chamber was flushed continuously with humidified air during the recording periods. The inlet and outlet tubes were temporarily clamped and pressure changes associated with each breath were recorded with a differential pressure transducer (Validyne MP, 45 ± 3 cm H_2O, Northridge, CA), connected to the animal and reference chambers. Whole body plethysmography requires the simultaneous measurements of pressure as well as ambient and rectal temperatures. The spirogram was recorded and stored on a computer with an acquisition data card (PCI-DAS1000, Dipsi, Chatillon, France) using respiratory acquisition software (Acquis 1 software, CNRS, Gif-sur-Yvette, France) for analysis off-line.

This technique was daily validated with a series of leak tests (leak was signalled by a diminution of the signal amplitude exceeding 33% in 5 s) (Bonora et al., 2004) and a series of calibrations performed by 10 injections of 100 μl of air into the chamber. The quantification threshold corresponded to a minimum air volume injection of 11 μl. Within the range of tested volumes (11–250 μl), measurements were linear. The mean coefficient of intra-day variability (four series of five measurements carried out the same day) was 1.3 ± 0.2%. The mean coefficient of inter-day variability (25 measurements carried out on 3 different days) was 1.7 ± 0.1%. We verified that the mean CO_2 measured using an Ohmeda 5250 RGM capnograph (rebreathing test) during clamping periods did not exceed 0.6% of the air contained in the chamber.

During experimentation, the first measurement was performed after a 60 min-period of accommodation, while the animal was quiet and not in deep or rapid eye movement sleep which can be roughly estimated from their behaviour, the response to noise, and the pattern of breathing. Then, the animal was gently removed from the chamber for i.p. injection, and replaced in the chamber for the remaining measurements. Ventilation was recorded at 5, 10, 15, 20, 30, 40, 60, 80, and 120 min, each record lasting about 60 s. The following parameters were measured: the tidal volume (V_T), the inspiratory time (T_I), the expiratory time (T_E), and the respiratory cycle duration ($T_{TOT} = T_I + T_E$). Additional parameters were calculated: the respiratory frequency (f), and the minute volume ($V_E = V_T \times f$).

2.4. Determination of P-gp expression in cerebral microvessels

Mouse brain microvessels were isolated as previously described (Yousif et al., 2007). Brains from three mice were pooled for each experiment. All procedures were carried out at 4 °C unless otherwise stated. Briefly, mice were euthanized by CO_2 inhalation and the brain immediately removed and placed in ice-cold Hank's Buffered Salt Solution (HBSS) + 10 mM HEPES (Isolation Buffer, IB). After removal of the cerebellums, meninges, brainstems, and large superficial blood vessels, brains were minced and homogenized with a Potter–Thomas homogenizer. The homogenate was centrifuged at 1000 × *g* for 10 min. The pellet was suspended in IB + 17.5% dextran and centrifuged for 15 min at 4400 × *g*. The supernatant containing a layer of myelin was discarded and the pelleted microvessels were suspended in IB + 1% bovine serum albumin (BSA), passed through a 20-μm nylon mesh and washed several times to eliminate cell debris. The fraction retained on this mesh was collected in IB + 1% BSA, centrifuged, and resuspended in lysis buffer containing 50 mM Tris–HCl pH 7.5, 50 mM NaCl, 30 mM sodium deoxycholate, 1% Triton X-100, and complete™ protease inhibitor complex (Roche Diagnostics, Meylan, France). After sonication, homogenates were centrifuged for 15 min at 10,000 × *g* and supernatants stored for western blot analysis.

The protein content of samples was determined using bicinchoninic acid assay (Pierce, Rockford, IL, USA). Equal amounts of protein (30 μg) were loaded on an 8% SDS-PAGE gel and electrophoresed at 120 mV for 90 min. Proteins were then transferred onto polyvinylidene difluoride membrane at 90 mV for 2 h using a wet electroblotting system (Biorad, Marnes-la-Coquette, France). After blocking in TBS containing 0.1% Tween 20 (TBST) and 5% non-fat milk (1 h at room temperature), the membrane was immunoblotted with mouse anti-P-gp antibody (C-219, Alexis Biochemicals, San Diego, USA) diluted 1:200, overnight at 4 °C. After several washings in TBST, the membrane was incubated in anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies (GE healthcare, Buckinghamshire, UK) diluted 1:10,000 for 1 h at room temperature. Chemiluminescent signals were revealed using the Immun-Star™ WesternC™ Chemiluminescent kit (Bio-Rad, France) and acquired with the ChemiDoc™ XRS imaging device (Bio-Rad, France). After careful rinsing in TBST, the membrane was reprobed with HRP-conjugated mouse anti-β-actin antibodies (ab49900, Abcam, Cambridge, UK) diluted 1:50,000 and the signals revealed as above. Signal intensity for each band was quantified using Image J software (National Institute of Health, Bethesda, MD, USA) and P-gp signals normalized upon actin signal.

2.5. In situ brain perfusion

BUP and NBUP transport at the BBB were measured in mice by *in situ* brain perfusion, as previously described (Cattelotte et al., 2008). The right common carotid artery was catheterized and used as site of perfusate entry. [^{3}H]-BUP (0.4 μCi/ml; 5 nM) or NBUP (15 μM) were added to the perfusion fluid (5 μM). [^{14}C]-Sucrose (0.1 μCi/ml) added to the perfusion fluid was used as a vascular space marker. Perfusion times were 30 s for [^{3}H]-BUP and 180 s for NBUP. Brain perfusion was terminated by decapitating the mouse at these selected times.

To measure BUP transport, radioactive samples from the right cerebral hemisphere were digested in 2 ml of Solvable (Perkin Elmer) at 50 °C and mixed with 9 ml of Ultima GoldXR (Perkin Elmer). Dual label counting was performed in a Packard Tri-Carb 1900TR. To measure NBUP transport, hydrochloric acid was added (2 ml/g of brain tissue), and the mixture was homogenized using sonication and stored at −80 °C before determination of NBUP concentrations by gas chromatography–mass spectrometry (GC–MS, internal standards: d4-BUP and d3-NBUP) (Alhaddad et al., 2012). The lower limits of quantification of the GC/MS method were 0.5 ng/ml for both BUP and NBUP.

The brain transport parameter K_{in} (μl/s/g) also called brain clearance was calculated as previously described (Cattelotte et al., 2008; Dagenais et al., 2000).

2.6. Study designs

2.6.1. Study 1: strain-related variations in BUP- and NBUP-induced dose/respiratory effects

Following randomization (N = 8/dose), male Fvb and Swiss mice were treated i.p. with either BUP (1, 10, 30, 45, and 60 mg/kg body weight), NBUP (1, 3, and 9 mg/kg body weight) or solvent (Tween 4%). Dose ranges were adapted from those