Psychoneuroendocrinology (2008) 33, 292–304

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Limited effects of β-endorphin compared to loperamide or fentanyl in a neuroendocrine biomarker assay in non-human primates

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Received 23 July 2007; received in revised form 4 October 2007; accepted 20 November 2007

KEYWORDS
β-Endorphin;
Prolactin;
Macaca;
Loperamide;
Biomarker;
Opioid

Summary
The *in vivo* pharmacodynamics of the opioid neuropeptide β-endorphin (a major endogenous agonist at the μ-opioid receptor) is difficult to determine in non-human primate models with translational value, or in humans. The present studies therefore employed a neuroendocrine biomarker assay, prolactin release, to systematically compare the *in vivo* profile of i.v. β-endorphin (0.01–0.32 mg/kg; i.v.) in gonadally intact male rhesus monkeys ($n = 4$) to that of the peripherally selective μ-agonist loperamide (0.01–0.32 mg/kg; i.v.) and the centrally penetrating μ-agonist fentanyl (0.0056–0.018 mg/kg; i.v.). Studies utilized a standardized time course design (measuring prolactin levels 5–120 min after agonist administration). β-Endorphin displayed only limited effectiveness in causing prolactin release when tested over this 30-fold dose range, compared to loperamide or fentanyl. Furthermore, two of the four subjects were only minimally responsive to β-endorphin. This differential responsiveness was not due to the presence of a previously described single nucleotide polymorphism at the *OPRM*1 gene (C77G), known to affect β-endorphin pharmacodynamics *in vitro*. *In vivo* biotransformation studies with MALDI-mass spectrometry determined that full-length β-endorphin was detectable in all subjects up to at least 5 min after i.v. administration. Thus, the relative ineffectiveness of

Abbreviations: β-End, β-endorphin; MALDI, matrix-assisted laser desorption/ionization mass spectrometry; *m/z*, mass/charge ratio; NLM, nalmefene; *OPRM*1, μ-receptor gene; PT, pretreatment; Δng/ml, change in serum prolactin levels from pre-injection baseline; Q-NTX, quaternary naltrexone; SNP, single nucleotide polymorphism.

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0306-4530/$ - see front matter
doi:10.1016/j.psyneuen.2007.11.011

i.v. β-endorphin in this assay does not appear to be principally due to rapid generation of non-opioid fragments of this neuropeptide.

1. Introduction

β-Endorphin (1–131) is an endogenous opioid neuropeptide derived from pro-opiomelanocortin. β-Endorphin is thought to act primarily as a μ-(MOP-r) agonist *in vitro*, and also has affinity at δ-receptors (Mansour et al., 1995). The actions of β-endorphin at μ-receptors *in vitro* are consistent with high efficacy agonism; β-endorphin also exhibits high propensity to cause μ-receptor desensitization and internalization (Alt et al., 1998; Beyer et al., 2004). In the periphery, certain effects of β-endorphin are detected, for example in the mediation of neuroendocrine and immune functions (Wardlaw et al., 1980; Rittner et al., 2001). There are few studies *in vivo* in humans or non-human primates that systematically compare the pharmacological profile of β-endorphin with that of non-peptidic μ-agonists. Studies in non-human primates suggest that systemically administered β-endorphin is unable to produce centrally mediated effects, presumably due to pharmacokinetic factors, including difficulty in crossing the blood–brain barrier (Domino and Li, 1985). Therefore, classic effects of μ-agonists (e.g., analgesia, respiratory depression) cannot be used to study β-endorphin's *in vivo* pharmacodynamics in translationally viable ways (e.g., by systemic administration).

A neuroendocrine biomarker such as prolactin release is one of the potential ways to study *in vivo* the μ-agonist effects of systemically administered β-endorphin in humans or non-human primates. For example, we have previously reported relatively potent and efficacious actions of the opioid peptide dynorphin A(1–17) in this biomarker (Butelman et al., 2004), consistent with its *in vitro* profile at κ-receptors (Zhu et al., 1997; Remmers et al., 1999). Prolactin release is under tonic inhibitory control by the hypothalamic tubero-infundibular system, which releases dopamine into the portal system (Moore and Lookingland, 1995). Dopamine acts at D_2-like receptors in anterior pituitary lactotroph cells, to maintain inhibition of prolactin release. The μ- and κ-opioid agonists cause prolactin release *in vivo*, presumably by modulating dopaminergic tubero-infundibular function (compounds from other pharmacological classes also cause prolactin release) (Blackford et al., 1992; Manzanares et al., 1992). Opioid receptor populations involved in this effect may be functionally outside the blood–brain barrier (Pechnik et al., 1987; Merchenthaler, 1991; Simpkins et al., 1991; Butelman et al., 1999b). However, μ-opioid receptors located within the blood–brain barrier may also modulate prolactin levels, in rodents (e.g., during stress) (Panerai et al., 1981; Odio and Brodish, 1990). To our knowledge, no studies in humans or non-human primates have determined whether the μ-receptors mediating this neuroendocrine effect are located outside the blood–brain barrier.

By contrast, δ-receptors (for which β-endorphin also has affinity) (Mansour et al., 1995) do not appear to mediate this neuroendocrine effect (Bero et al., 1987; Butelman et al., 2002). Given β-endorphin's binding profile and potential peripheral selectivity, this neuroendocrine endpoint could therefore be a potentially useful *in vivo* biomarker for its μ-agonist effects.

Pioneering studies in humans determined that i.v. β-endorphin (like non-peptidic μ-agonists) caused prolactin release (Foley et al., 1979; Lehmann et al., 1979; Catlin et al., 1980). Most of these studies used single β-endorphin doses in normal volunteers, or in patients with different ongoing clinical conditions. Furthermore, the pharmacological profile of β-endorphin could not be systematically compared to that of non-peptidic reference μ-agonists. A study in rhesus monkeys determined that i.v. β-endorphin (probed over a two-fold dose range: 0.01–0.02 mg/kg) resulted in prolactin release; this limited dose range precluded a systematic comparison to a non-peptidic μ-agonist (Gilbeau et al., 1985; see also Spies et al., 1980). Another pioneering study in non-human primates confirmed that the likely mode of action of β-endorphin was through hypothalamic opioid receptors modulating the tubero-infundibular system (Wardlaw et al., 1980).

The *in vivo* pharmacodynamics of β-endorphin is of interest because it is a major endogenous ligand for μ-receptors, the target of major analgesics as well as of prominent drugs of abuse. For example, it is of value to determine whether β-endorphin acts as a high efficacy agonist *in vivo* (as predicted by its *in vitro* profile), and to determine individual subject responsiveness to this neuropeptide. As an illustration of this latter point, a functional single nucleotide polymorphism (SNP) in humans (A118G), and an ortholog in rhesus monkeys (C77G) exhibit an increased potency of β-endorphin in cell culture systems (Bond et al., 1998; Miller et al., 2004). These SNPs also have relevance to functions thought to be modulated by μ-receptors, including drug and alcohol addiction, stress responsiveness and analgesia (Lotsch et al., 2004; Bart et al., 2005; Barr et al., 2007; Chong et al., 2006).

A complicating factor in the study of systemic β-endorphin effects is the potential biotransformation of this neuropeptide, yielding active or inactive fragments (Mansour et al., 1995; Sandin et al., 1998). Few studies have characterized the *in vivo* biotransformation of systemic β-endorphin *in vivo* with mass spectrometry techniques. Such techniques have the advantage of positively identifying fragments of this neuropeptide, given the challenges of identification using antibody-based approaches (Schulz et al., 2006; Babu et al., 2006).

In these studies, we therefore systematically compared the pharmacological profile of i.v. β-endorphin with a peripherally selective μ-agonist, loperamide, and with a centrally penetrating μ-agonist, fentanyl, in male rhesus monkeys characterized for the potential SNP of the μ-opioid receptor (C77G). We also delineated the basic biotransformation profile of β-endorphin *in vivo* using MALDI-mass spectrometry, incorporating novel sample preparation techniques.

2. Methods

2.1. Experimental subjects in neuroendocrine studies

Four captive-bred, gonadally intact male rhesus monkeys (*Macaca mulatta*; age range: 8–11 years old approximately; weight range: 9.0–12.5 kg), were used. Monkeys were singly housed in a room maintained at 20–22 °C with controlled humidity, and a 12-h light:12-h dark cycle (lights on at 07:00 h). Monkeys were fed approximately 12 jumbo primate chow biscuits (PMI Feeds, Richmond, VA) daily, supplemented by appetitive treats, and multivitamins plus iron. An environmental enrichment plan was in place in the colony rooms (e.g., music, nature sounds and videos). Water was freely available in home cages, via an automatic waterspout.

These studies were reviewed by the Rockefeller University Animal Care and Use Committee, in accordance with the Guide for the Care and Use of Animals (National Academy Press, Washington, DC, 1996).

2.2. Single nucleotide polymorphism analysis for *OPRM*1 (μ-receptor) gene

Genomic DNA was isolated from peripheral white blood cells, obtained by venipuncture from all the subjects used in the neuroendocrine experiments (Versagene kit, Gentrasystems, Minneapolis, MN). Exon 1 of the *M. mulatta OPRM*1 was amplified by PCR, using a forward primer (5′-CCACGAACGCCAGCAATT-3′) and a reverse primer (5′-CACGCACACGATG GAGTAGA-3′), located 20 base pairs downstream and 227 base pairs downstream of translation initiation codon ATG, respectively (GenBank accession no. AF286024). To identify SNPs, PCR products, 227 base pairs in size, were sequenced in both directions using the Big Dye Terminator Cycle Sequencing Kit (ABI, Applied Biosystems, Foster City, CA) and an ABI Prism 3700 capillary sequencer.

2.3. Procedure for neuroendocrine experiments

Chair-trained monkeys were tested after extensive prior exposure to the experimental situation. Monkeys were chaired and transferred to the experimental room between 10:00 and 11:00 h on each test day. An indwelling catheter (24 gauge; Angiocath, Becton Dickinson, Sandy, UT) was placed in a superficial leg vein, and secured with elastic tape. An injection port (Terumo, Elkton, MD) was attached to the hub of the catheter; port and catheter were flushed (0.3 ml of 50 U/ml heparinized saline) before use, and after each blood sampling or i.v. injection. Approximately 20 min following catheter placement, two baseline blood samples of approximately 1.5 ml were collected, 5 min apart from each other (defined as −10 and −5 min, relative to the onset of dosing), and kept at room temperature until the time of spinning (3000 rpm at 4 °C) and serum separation. Serum samples were then kept at −40 °C until the time of analysis; typically within 2 weeks of collection. The samples were analyzed in duplicate with a standard human prolactin immunoradiometric kit (DPC, Los Angeles, CA), following manufacturer's instructions. There is high protein homology between human and rhesus monkey prolactin, and antibody cross-reactivity between human and rhesus monkey prolactin has also been reported (Brown and Bethea, 1994; Pecins-Thompson et al., 1996). The reported sensitivity limit of this assay was 0.1 ng/ml; each individual kit was calibrated with known standards, in the range 2–200 ng/ml. The intra- and inter-assay coefficients of variation with this kit in the laboratory were approximately 2% and 14%, respectively. Monkeys were tested in a time course procedure. Following baseline sample collection, a single agonist dose (i.e., of β-endorphin, loperamide or fentanyl) was administered i.v., followed by sampling at 5, 15, 30, 60, 90 and 120 min after administration. In antagonism experiments, a single dose of antagonist (s.c. quaternary naltrexone or nalmefene) was administered 30 min before an agonist, followed by testing as above. In these antagonism experiments, a single sample was also taken 20 min after administration of the antagonist alone (i.e., during the pretreatment period). Consecutive experiments in the same subject were separated by at least 72 h; the order of experiments was unsystematic among subjects. All studies were carried out over the course of several months, while all subjects were in stable colony rooms.

2.4. Design

Each experiment was carried out with $n = 4$, unless otherwise stated. In specific cases (especially antagonism pretreatment studies to loperamide), one subject (MAR) was not studied, due to its low responsiveness to loperamide alone (e.g., Table 1).

The prolactin-releasing effects of i.v. vehicle, β-endorphin (0.01–0.32 mg/kg), loperamide (0.032–0.32 mg/kg) and fentanyl (0.0056–0.018 mg/kg) were initially investigated. This was followed by antagonism experiments, with single pretreatment doses of quaternary naltrexone (0.32 or 1 mg/kg; s.c.) administered before the largest dose of loperamide studied above (0.32 mg/kg; $n = 3$). The larger dose of quaternary naltrexone (1 mg/kg) was also studied as a pretreatment to fentanyl (0.018 mg/kg; $n = 4$). A nalmefene dose (0.01 mg/kg) previously shown to antagonize μ-receptor mediated effects was also studied as a pretreatment to fentanyl (0.018 mg/kg) (France and Gerak, 1994; Butelman et al., 2002). In order to determine whether β-endorphin's relative lack of activity in this assay was due to partial agonist-like actions at μ-receptors, one of the maximally effective β-endorphin doses (0.1 mg/kg, i.v.), was administered as a 5 min pretreatment to loperamide (0.32 mg/kg; i.v.; $n = 3$). A partial agonist would be predicted to block the actions of a higher efficacy agonist, in an endpoint in which the former is not active in itself (Kenakin, 1993; Gerak et al., 1994).

2.5. Neuroendocrine data analysis

Raw individual prolactin values were converted to Δng/ml (i.e., absolute change from baseline) by subtracting the mean pre-injection value for each experiment in each subject. Data were then analyzed with two-way repeated measures ANOVAs (e.g., time × drug condition) using Sigmastat 3.1, followed by Newman–Keuls post hoc testing,

Table 1 Peak effects for the largest doses of β-endorphin, loperamide and fentanyl in individual subjects, compared to vehicle (Δng/ml)*

Subject ID	Vehicle	β-Endorphin (0.32 mg/kg)	Loperamide (0.32 mg/kg)	Fentanyl (0.018 mg/kg)
NAT	−1.0	7.6	57.3	65.8
MAR	−0.3	1.2	3.7	76.0
NIC	−4.5	26.8	127.9	261.2
T9V	−2.7	18.9	162.2	116.3

*Individual subject data (Δng/ml) are presented for vehicle and the largest dose of each agonist studied herein, at a time of peak effect (15 min post-administration; see Figure 1).

as appropriate. The level of significance (α) for all comparisons was set at the 0.05 level.

2.6. β-Endorphin biotransformation studies

2.6.1. *Ex vivo* biotransformation studies

Approximately, 4 ml of blood was obtained in a heparinized vacutainer, and stored on ice for less than 30 min prior to experiment ($n = 4$). Blood was separated into 100 μl aliquots for each time-point of interest, in triplicate. β-Endorphin (MW = 3465) was added to give a final concentration of 1 μM (corresponding to 0.0035 mg/ml), and the blood was incubated for the indicated time.

2.6.2. Selection of *ex vivo* β-endorphin concentration

A mid-range estimate of total blood volume in this species, from the literature, is 73 ml/kg body weight (Fortman et al., 2002). Therefore, the selected *ex vivo* β-endorphin concentration is an estimate of the theoretical maximum concentration observed immediately after an i.v. bolus of 0.26 mg/kg (i.e., 0.0035 mg/ml concentration × 73 ml/kg body weight = 0.26 mg/kg body weight) (Rowland and Tozer, 1980). Thus, these *ex vivo* studies used a β-endorphin concentration theoretically within the range of *in vivo* β-endorphin doses in the neuroendocrine studies herein (i.e., 0.01–0.32 mg/kg, i.v.). Blood aliquots were centrifuged (2 min, 5000 rpm); 40 μl plasma was removed and added to microcentrifuge tubes containing 2 μg C18-coated magnetic beads (Dynal, Invitrogen, Carlsbad, CA) in 10 μl of 0.1% trifluoroacetic acid (TFA), containing 400 fmol of internal standard, thymopoietin fragment II (m/z 1610.8), also used as a mass calibrant (Sigma, St. Louis, MO). The beads were then isolated via magnetic application, and the plasma removed. The beads were washed three times with 25 μl of 0.1% TFA. The absorbed peptides were eluted directly from the beads with a saturated solution of HCCA diluted six-fold with 1 μl of 2:1 acetonitrile:water (0.1% TFA), and spotted directly on the MALDI plate.

2.6.3. *In vivo* biotransformation

The *in vivo* procedure is similar to that used in the above neuroendocrine studies, other than for the timing of the obtained samples. These biotransformation sessions (β-endorphin 0.1 mg/kg i.v.; $n = 4$) were carried separately in each of the above subjects. In biotransformation sessions, one catheter was placed in each leg. One catheter was used for injection, and only the contralateral catheter was used for post-injection sampling. A pre-injection sample was obtained, followed by β-endorphin bolus injection (approximately 30 s in duration with 1 mg/ml solution), followed by catheter flushing. Samples were then obtained at time 0, 1, 2, 5, 15 and 30 min after injection (time 0 being immediately following the end of injection and flushing). Blood samples (1.5 ml each) were placed in chilled heparinized vacutainers, and were kept on ice until spinning and plasma separation.

Assays were conducted in triplicate, by incubation of 50 μl of plasma with 2 μg C18-coated magnetic beads (Dynal) in 10 μl of 0.1% TFA containing 400 fmol of thymopoietin fragment II (m/z 1610.8) and neuropeptide Y (m/z 4272.7), which were also used as mass calibrants (Phoenix Pharmaceuticals; Burlingame, CA). The beads were then isolated via magnetic application, and the plasma removed. The beads were washed three times with 25 μl of 0.1% TFA. The absorbed peptides were eluted directly from the beads with a saturated solution of HCCA diluted six-fold with 1 μl of 2:1 acetonitrile:water (0.1% TFA), and spotted directly on the MALDI plate.

2.6.4. MALDI-mass spectrometry technique (*ex vivo* or *in vivo* biotransformation samples)

For MALDI time-of-flight mass spectrometric analysis, a Voyager-DE STR Mass Spectrometer was used in linear mode, with delayed extraction. Each spectrum represents a sum of 150–200 laser shots, and is smoothed and calibrated using the program M-over-Z (Genomic Solutions, Lansing, MI). Relative normalization of the heights of the relevant β-endorphin peaks in the spectra, to internal standard or to a specific constitutive peak in plasma, was performed using Origin C and Origin Labview software (Microcal, Northampton, MA). Results for each time-point are averaged across three replicates of a sample, and normalized to the relative peak height at time-point zero for each subject, to afford relative quantification of β-endorphin (1–31) levels over post-injection time.

2.6.5. Drugs

Synthetic human β-endorphin (NeoMPS, San Diego, CA; kindly supplied through the NIH-NIDA Drug Supply program) was dissolved in sterile saline approximately 10 min before use. Fentanyl citrate (Sigma, St. Louis, MO) was dissolved in sterile water. Quaternary naltrexone (naltrexone methobromide, also known as methylnaltrexone; kindly supplied by Dr. Chun-su Yuan; Department of Anesthesiology, University

of Chicago, Chicago, IL) was dissolved in sterile water approximately 10 min before use. Loperamide HCl (Sigma, St. Louis, MO) was dissolved in a vehicle composed of 10% ethanol, 10% Tween 80 and 80% sterile water, by volume. Drug injections were made in volumes in the range of 0.05–0.1 ml/kg, whenever possible. All doses of compounds are expressed in the forms mentioned above.

3. Results

3.1. *OPRM*1 single nucleotide polymorphisms

Sequence analysis of the N-terminal of the rhesus monkey *OPRM*1 revealed that all the subjects in the present neuroendocrine studies were homozygotes for the common C77 allele; thus none of these subjects had the previously reported C77G SNP (Miller et al., 2004). Interestingly, a previously unreported SNP, G86C, was detected in one of the present subjects (subject NIC, a heterozygote at this locus). This new SNP would result in a predicted change in a glycine residue to alanine at position 29.

3.2. Baseline prolactin levels and effect of vehicle administration

Baseline prolactin levels were relatively stable across sessions. In a vehicle control study, the mean pre-injection serum prolactin levels for these subjects was 10.4 ng/ml (S.E.M. = 2.9). Small gradual decreases in serum prolactin levels were observed from 5 to 120 min after vehicle injection (Figure 1). For example, at a time of peak effects for the agonists in this study (15 min after administration; see Figure 1), vehicle administration resulted in a small decrease from baseline of −2.1 Δng/ml (S.E.M. = 0.9) (Table 1).

3.3. Effects of β-endorphin, loperamide and fentanyl on prolactin levels

β-Endorphin, probed over a 30-fold dose range (0.01–0.32 mg/kg) resulted in small increases in prolactin levels, as detected 5, 15 and 30 min after administration (Figure 1). A two-way (time × dose) repeated measures ANOVA for β-endorphin and vehicle (i.e., 4 β-endorphin doses and vehicle) detected a significant main effect of time ($F[5,15] = 5.56$), and a significant time × dose interaction ($F[20,60] = 1.96$). Newman–Keuls post hoc tests revealed that the effect of β-endorphin (0.1 and 0.32 mg/kg) were significantly different from vehicle 5, 15 and 30 min after administration.

Overall, two of the subjects (NIC and T9 V) exhibited the more robust dose-dependent effects of β-endorphin. Effects in the other two subjects (MAR and NAT) were less clearly detectable (see Figure 2 and Table 1 for individual data). Larger doses of β-endorphin were not probed in order to conserve supply, however, an apparent plateau is observable in the mean dose–effect curve at the two largest doses studied (0.1–0.32 mg/kg).

Loperamide (0.032–0.32 mg/kg) resulted in robust dose-dependent prolactin release in three of four subjects, with subject MAR being a ''non-responder'' (see Figures 1 and 2; Table 1). For the three other subjects, the largest dose of loperamide produced effects that were at least four-fold greater than those observed after the largest dose of β-endorphin (Table 1). Larger doses of i.v. loperamide were not probed to avoid previously reported potential untoward effects (Yanagita et al., 1979). A two-way (time × dose) repeated measures ANOVA detected main effects of time ($F[5,15] = 5.33$), dose ($F[3,9] = 4.76$) and their interaction ($F[15,45] = 2.41$). One data point (at +30 min) for one subject (T9V) was missing at the loperamide 0.1 mg/kg dose. This was replaced by an unbiased estimate, for analysis (the mean of the subject's data at the time-points immediately preceding and following the missing data point).

The largest dose of loperamide (0.32 mg/kg) was also studied after 5 min pre-treatment with β-endorphin (0.1 mg/kg, i.v.), in three subjects, excluding the loperamide ''non-responder'' (subject MAR; see Table 1). This study was designed in order to detect potential partial agonist-like effects of β-endorphin, given its low effectiveness alone (see above). This β-endorphin pretreatment did not alter the neuroendocrine effects of loperamide (0.32 mg/kg; Figure 3), under these conditions.

Fentanyl (0.0056–0.018 mg/kg) resulted in robust dose-dependent prolactin release in all four subjects (Figures 1 and 2). A two-way (time × dose) repeated measures ANOVA detected main effects of time ($F[5,15] = 9.88$) dose ($F[2,6] = 5.45$), and their interaction ($F[10,30] = 11.54$). The largest dose of fentanyl (0.018 mg/kg) produced effects that were at least eight-fold greater than those observed with the largest dose of β-endorphin (0.32 mg/kg), in each subject (Table 1). Larger doses of i.v. fentanyl were not studied, to avoid known respiratory depressant effects (Ko et al., 2002).

3.4. Antagonism experiments

The largest dose of loperamide (0.32 mg/kg) was studied after pretreatment with quaternary naltrexone, in the three subjects that exhibited an effect with loperamide alone (subject MAR was thus excluded from study; see Table 1). Quaternary naltrexone (0.32–1 mg/kg) caused dose-dependent and full blockade of the effects of loperamide (0.32 mg/kg; Figure 4). A two-way (time × quaternary naltrexone pretreatment condition) repeated measures ANOVA revealed a main effect of time ($F[5,10] = 8.63$), quaternary naltrexone pretreatment ($F[2,4] = 12.28$) and their interaction ($F[10,20] = 10.54$). No obvious effects were observed when a sample was taken 20 min after quaternary naltrexone alone (i.e., during the pretreatment period). In Newman–Keuls post hoc comparisons, pretreatment with either dose of quaternary naltrexone (0.32 or 1 mg/kg) was significantly different from loperamide (0.32 mg/kg) alone.

The largest dose of fentanyl (0.018 mg/kg) was also studied after the larger dose of the quaternary naltrexone (1 mg/kg; $n = 4$). This pretreatment produced substantial, but not complete antagonism of fentanyl (Figure 4). Larger doses of quaternary naltrexone were not studied, due to supply limitations. As a follow-up, the centrally penetrating

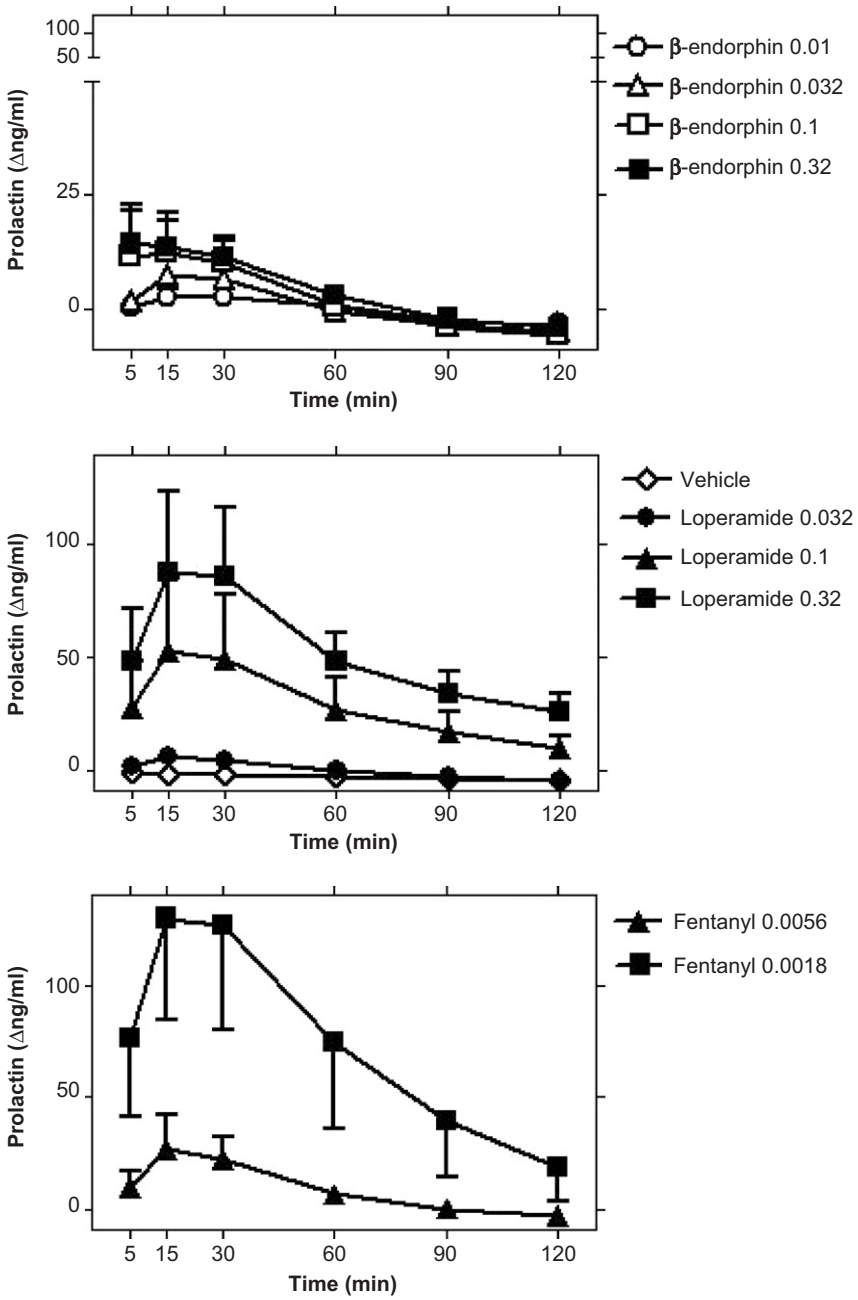

Figure 1 Time course of the effects of i.v. β-endorphin (0.01–0.32 mg/kg; upper panel), vehicle or loperamide (0.032–0.32 mg/kg; middle panel) and fentanyl (0.0056–0.018 mg/kg; lower panel) on serum prolactin levels. Data are mean ± S.E.M. ($n = 4$; same subjects used in all studies). Abscissae (all panels) time from i.v. injection. Ordinates (all panels) change in serum prolactin levels (Δng/ml), from the individual pre-injection baseline of each subject (mean of two baseline samples; −10 and −5 min). Note ordinate axis break and axis range in lower panel.

antagonist nalmefene (0.01 mg/kg, s.c.) was also studied as a pretreatment to fentanyl (0.018 mg/kg). This nalmefene dose caused complete blockade of the effects of fentanyl in this assay (Figure 4). A two-way (time × pretreatment condition; i.e., no pretreatment, quaternary naltrexone or nalmefene) repeated measures ANOVA revealed a significant effect of time ($F[5,15] = 10.44$), pretreatment condition ($F[2,6] = 5.86$) and a significant interaction between time and pretreatment condition ($F[10,30] = 9.48$). Post hoc analyses show that either quaternary naltrexone or nalmefene were significantly different from the no pretreatment condition (with Newman–Keuls comparisons at 15, 30 and 60 min after fentanyl administration).

In a pilot study, β-endorphin (0.1 mg/kg, i.v.) was studied after quaternary naltrexone (1 mg/kg, s.c.) pretreatment in the two subjects that exhibited the most robust β-endorphin-induced effects (NIC and T9V; see Figure 2; Table 1). In both these subjects, this quaternary naltrexone pretreatment fully blocked the effects of β-endorphin. Thus, β-endorphin alone (0.1 mg/kg) at a time of peak effect

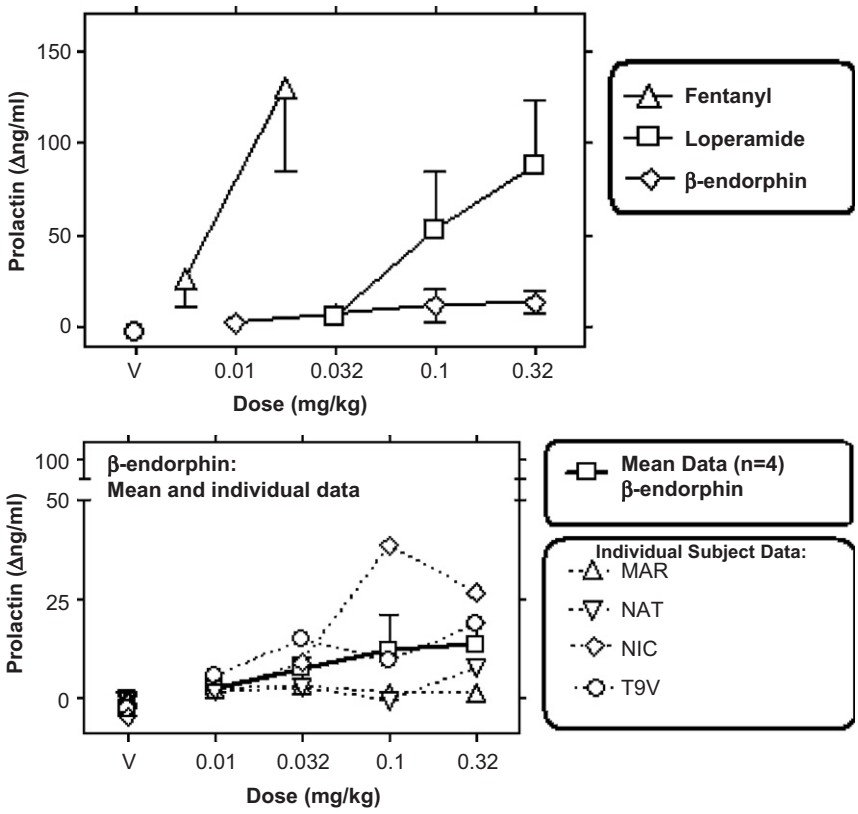

Figure 2 Dose–effect curves for β-endorphin, loperamide and fentanyl, re-plotted from a time of peak effect (15 min post-administration). Upper panel: Mean dose–effect curves ($n = 4$) for each aforementioned compound. Lower panel: Individual subject dose–effect curves for β-endorphin ($n = 4$; re-plotted from above panel). Abscissae are doses in mg/kg (points above "V" represent values obtained after vehicle administration); other details as in Figure 1. Note ordinate axis break and axis range in lower panel.

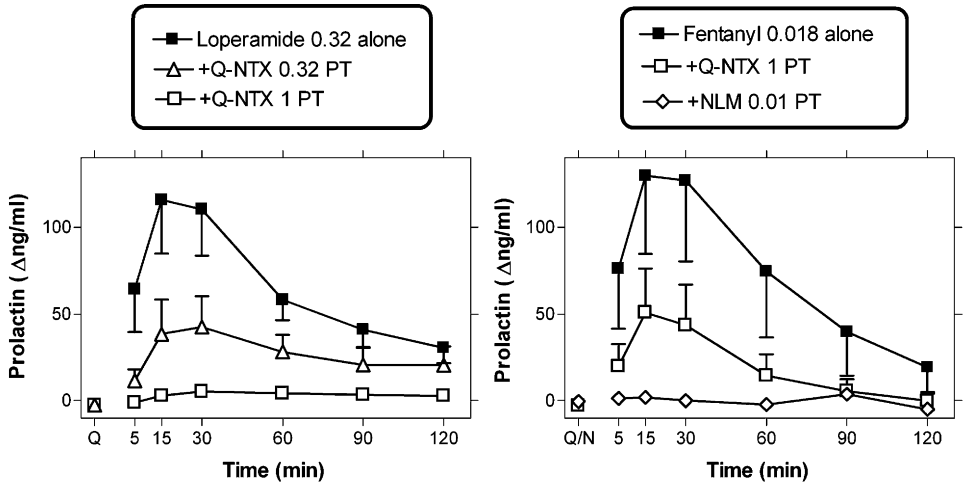

Figure 3 Left panel: Antagonism by quaternary naltrexone (0.32 or 1 mg/kg; s.c.) pretreatment (PT) to loperamide (0.32 mg/kg; i.v.). Data are $n = 3$; subject MAR was excluded from study due to its lack of response to loperamide alone (see Table 1). Point above "Q" was obtained 20 min after quaternary naltrexone alone. Right panel: Antagonism by quaternary naltrexone (1 mg/kg; s.c.) or nalmefene (0.01 mg/kg; s.c.) of the effects of fentanyl (0.018 mg/kg; i.v.; $n = 4$). Point above "Q/N" was obtained 20 min after quaternary naltrexone or nalmefene alone. Other details as in upper panel and Figure 1.

(e.g., 15 min after administration) caused changes in prolactin levels of +38.8 and +9.4 Δng/ml in NIC and T9V, respectively (see Figure 2). After pretreatment with quaternary naltrexone (1 mg/kg), this effect of β-endorphin was completely blocked, resulting in −4.3 and −5 Δng/ml for NIC and T9V, respectively. For comparison, these latter values are indistinguishable from the effects of i.v. vehicle at this time-point (e.g., Table 1). The two other subjects (MAR and NAT) were not studied, due to the small magnitude of their response to β-endorphin alone (e.g., Table 1).

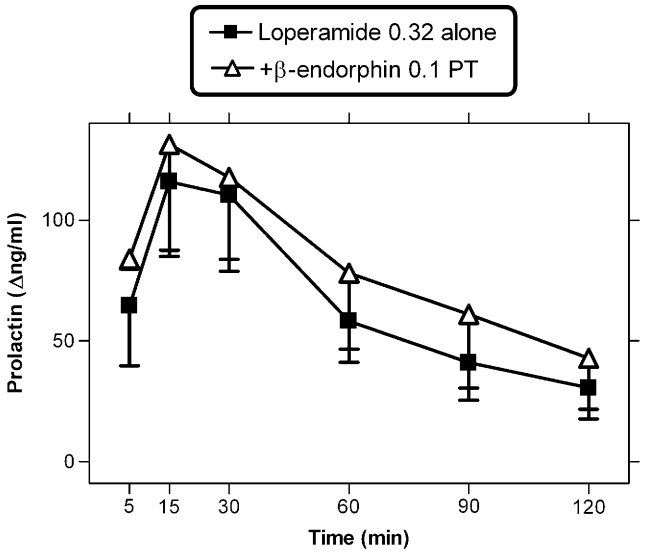

Figure 4 β-Endorphin (0.1 mg/kg; i.v.) pretreatment administered 5 min before loperamide (0.32 mg/kg; i.v.). Data are $n = 3$; subject MAR was excluded due to lack of response to loperamide alone (see Table 1).

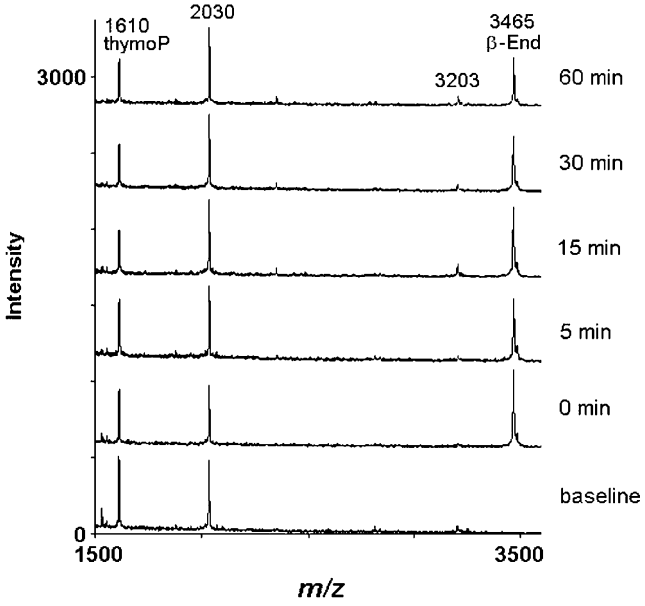

Figure 5 *Ex vivo* biotransformation of 1 μM β-endorphin incubated in blood at 37 °C. Representative mass spectra are shown for a single subject, following *ex vivo* incubation of β-endorphin for the indicated times. A baseline sample was obtained prior to the addition of β-endorphin. Spectra were obtained in linear mode, with a *m/z* (mass/charge) range from 1000 to 10,000, and represent averages of 150–200 laser shots. For clarity, spectra were truncated to a *m/z* range of 1500–3600. The standard thymopoietin B (ThymoP; *m/z* 1610) was added to the magnetic C18 beads used for solid-phase extraction. For each time-point, at least three replicates were processed; mass spectra shown represent a single replicate each. Abscissa: mass/charge; ordinate: peak intensity.

3.5. Biotransformation

3.5.1. *Ex vivo* biotransformation

The possibility that the relatively low prolactin release response to β-endorphin was due to rapid degradation of the peptide was explored. We incubated β-endorphin *ex vivo* in blood of each of the present subjects at 37 °C ($n = 4$). We did not observe identifiable cleavage products recoverable in plasma, when β-endorphin (1 μM) was incubated for up to 1 h in blood (see representative mass spectra; Figure 5). Moreover, with respect to an external standard (thymopoietin B, which was also used as a mass calibrant), the levels of β-endorphin did not show a major decline over this *ex vivo* incubation period. There were several background peaks observed in the mass spectra, but these peaks were not of the same *m/z* as β-endorphin or any of its likely fragments. These background peaks were largely stable over time and observed in all animals tested (e.g., peak *m/z* 3203) (Figure 5).

3.5.2. *In vivo* biotransformation

Given the stability of β-endorphin *ex vivo*, we tested whether we could detect β-endorphin and potential cleavage products after *in vivo* administration of β-endorphin (0.1 mg/kg; i.v.), in each of the present subjects. We detected β-endorphin levels above baseline for at least 5 min after administration in each of the subjects (Figure 6). Normalization of β-endorphin to externally added standard (e.g., thymopoietin B) resulted in higher variability compared to normalization to the level of a peak from the plasma peak of *m/z* 3203; the former was therefore not used herein (see below).

As in the case of the *ex vivo* experiments, we observed several peaks in the baseline plasma samples; these peaks were stable over the time course of the experiment, and their *m/z* did not correspond to β-endorphin or any likely fragments thereof. In the case of a peak of *m/z* 3203, we observed it in all animals and it was stable. Using LC–MS–MS with electron transfer dissociation, we were able to obtain substantial sequencing information, and identified this baseline peak as a peptide fragment of plasma kallikrein-sensitive glycoprotein (amino acid residues 617–644; NVHSGSTFFRYYLQGAKMPKPEASFSPR). Due to its stability and relatively similar mass to β-endorphin (*m/z* 3465), we used the ratio of the β-endorphin peak to the peak for this plasma peptide (*m/z* 3203), for relative quantitation (Figure 7). We also detected two N-terminally truncated fragments of β-endorphin, 2–31 and 4–31. These fragments are not predicted to have opioid receptor affinity due to the lack of the Tyr^1 residue, and were present at low levels compared to the initial levels of full-length β-endorphin, in each subject. The half-life of full-length β-endorphin, calculated by an exponential decay fit to mean data ($n = 4$) was estimated to be 7.5 min.

4. Discussion

In this study, β-endorphin caused relatively small increases in serum prolactin in gonadally intact adult male rhesus

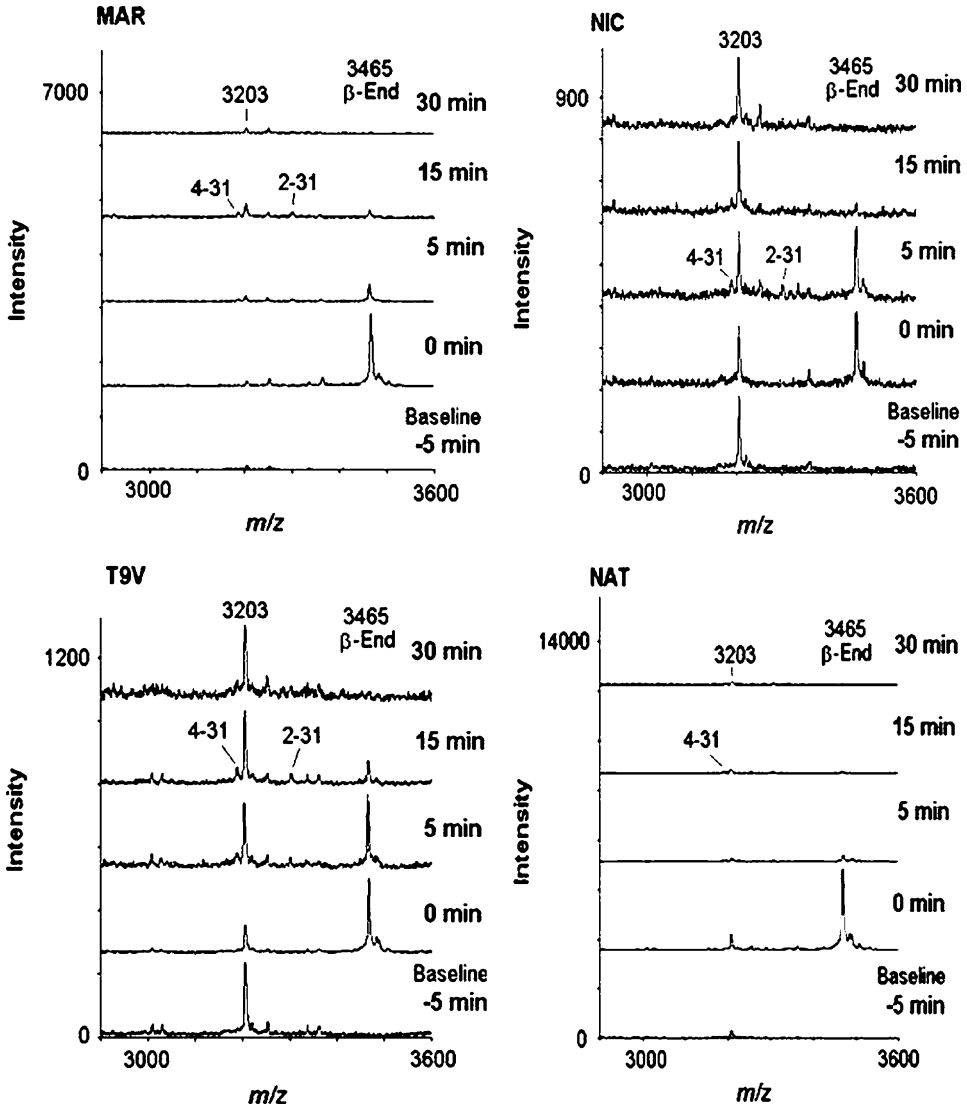

Figure 6 *In vivo* biotransformation of i.v. β-endorphin (0.1 mg/kg): representative mass spectra for each subject ($n = 4$). The baseline sample was obtained 5 min prior to β-endorphin administration, and remaining samples were obtained 0, 5, 15 and 30 min after administration, where time 0 was immediately after β-endorphin injection. For clarity, spectra at 1 and 2 min are omitted from this figure. Spectra were obtained in linear mode, with a *m*/*z* range of 1000–10,000, and represent averages of 150–200 laser shots. For clarity, spectra were truncated to a *m*/*z* range of 2900–3600. For data processing, involving peak normalization (see Figure 7), at least three technical replicates for each time-point were analyzed; mass spectra shown represent a single replicate each. Abscissa: mass/charge; ordinate: peak intensity.

monkeys. The maximum effect of β-endorphin, studied over a 30-fold range, was smaller than that observed with the other μ-agonists studied herein (loperamide and fentanyl), within each subject. In a pilot study in the two subjects that were relatively responsive to β-endorphin, the peripherally selective antagonist quaternary naltrexone fully blocked β-endorphin's effects. The maximum β-endorphin dose injected in this study (0.32 mg/kg) would be estimated to result in a maximum theoretical blood concentration of 4 μg/ml, immediately after i.v. bolus injection (assuming 73 ml blood/kg body weight; see Section 2). This concentration is at least 3 orders of magnitude greater than baseline blood β-endorphin concentrations in humans or macaques, reported in the literature with a variety of techniques (Wiedemann et al., 1979; Kalin et al., 1980; Dent et al., 1981; Kreek et al., 1983; Crockett et al., 2007). Therefore, comparatively limited neuroendocrine effect of β-endorphin in the present studies is unlikely to be due to insufficient dosing with this peptide.

Inter-subject differences in maximal β-endorphin effects were also observed, but the present dose–effect curve evaluation showed a plateau in the maximal effects of β-endorphin (e.g., at 0.1–0.32 mg/kg). One potential source of this variability could be *OPRM*1 genotype, since it is known that a C77G SNP of the rhesus monkey *OPRM*1 has an increased *in vitro* potency of β-endorphin as a phenotype (Miller et al., 2004). This non-human primate SNP is a functional ortholog of the widely studied A118G human SNP, thus is of potential translational value (Bond et al., 1998). However, variability in β-endorphin's neuroendocrine effect

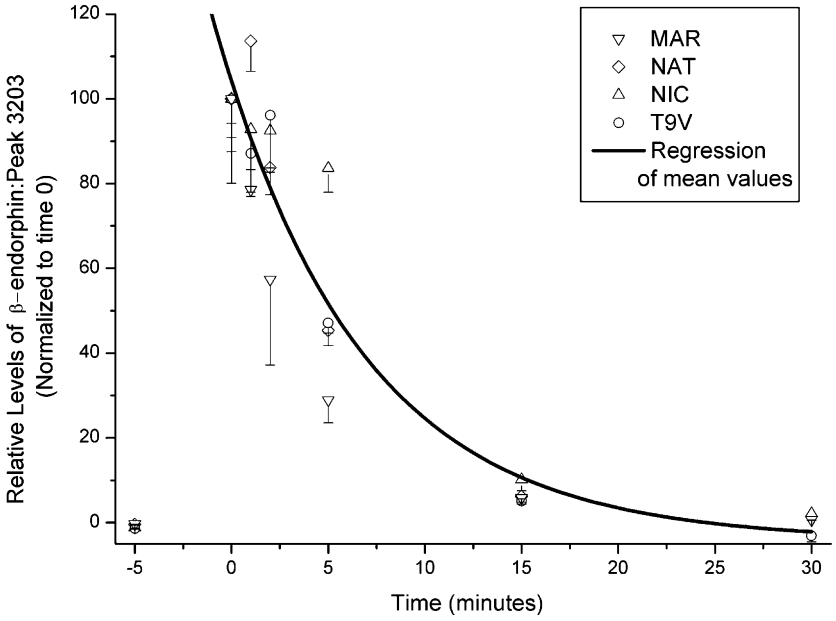

Figure 7 *In vivo* biotransformation of i.v. β-endorphin (0.1 mg/kg): time course of full-length β-endorphin peak ($n = 4$). Abscissa: time of sample (min). Ordinate: Relative peak levels of β-endorphin, expressed as a ratio of peak levels for a constitutive peptide present stably in blood (*m/z* 3203; see Figure 6). This peptide was sequenced using LC–MS–MS with electron transfer dissociation and shown to be a fragment of plasma kallikrein-sensitive glycoprotein (see Section 3). Data were normalized for the ratio of β-endorphin to peak 3203 present at time 0 for each subject. Error bars (S.E.M.) reflect variation in three solid-phase extraction/MALDI-MS replicates of the same blood sample. An exponential regression was calculated for mean data ($n = 4$).

in these subjects could not be accounted for by this *OPRM*1 SNP (C77G), since all were found to be homozygotes for the common C allele at this locus (Miller et al., 2004). Direct comparison to subjects having at least one G allele at this locus (i.e., CG heterozygotes or GG homozygotes) could in the future determine whether this SNP is associated with a change in pharmacodynamics of β-endorphin, with this biomarker.

The peripherally selective, non-peptidic μ-agonist loperamide caused robust dose-dependent effects in this neuroendocrine assay (in three of four subjects); these were fully prevented by the peripherally selective opioid antagonist, quaternary naltrexone. This confirms that in primates, μ-agonist induced prolactin release can be mediated by sites located functionally outside the blood–brain barrier (Wardlaw et al., 1980; Zheng et al., 2005). Prior studies, primarily in rodents, report that μ-receptor mRNA or protein can be detected in areas involved in the control of prolactin release, including the arcuate nucleus, and median eminence (e.g., Beauvillain et al., 1992; Zheng et al., 2005). These are also the first studies to detect a prolactin-releasing effect of loperamide *per se*, in humans or non-human primates, to our knowledge (e.g., at i.v. doses 0.1–0.32 mg/kg, herein). A prior study in humans (receiving 8 mg of loperamide orally; approximately equivalent 0.1 mg/kg in a 70 kg subject) did not detect such effects (Caldara et al., 1981). A possible difference between these two studies, aside from species, is that the oral dose of loperamide in the above human study was insufficient to achieve active systemic concentrations, in view of this compound's limited oral bioavailability (Heykants et al., 1974). Overall, the comparative loperamide experiments suggest that the postulated peripheral selectivity of systemically administered β-endorphin (Domino and Li, 1985), would not in itself render this neuropeptide inactive in this biomarker assay.

The largest fentanyl and loperamide doses studied herein were limited partially by safety considerations (Yanagita et al., 1979; Ko et al., 2002), and a plateau in these two compounds' effects was not observed over their respective dose ranges (in contrast to effects of β-endorphin). This is consistent with the conclusion that β-endorphin caused smaller maximal effects than either non-peptidic compound in this assay. Prior studies with κ-opioids in this assay show that opioid agonists with lower efficacy (e.g., partial agonists) tend to show lower maximal effects than higher efficacy agonists (Butelman et al., 1999a). However, *in vitro* studies support the conclusion that β-endorphin, like loperamide and fentanyl, acts as a relatively high efficacy agonist at μ-receptors (Selley et al., 1997; Alt et al., 1998; DeHaven-Hudkins et al., 1999). Furthermore, β-endorphin (0.1 mg/kg), given as a short (5 min) pretreatment to the largest loperamide dose (0.32 mg/kg), did not decrease loperamide's effects. Taken together, these findings suggest that low pharmacodynamic efficacy at μ-receptors (e.g., partial agonism) is unlikely to underlie β-endorphin's low effectiveness in this neuroendocrine assay. However, it is possible that specific pharmacodynamic properties of β-endorphin at μ-receptors (e.g., its greater propensity to cause receptor desensitization or endocytosis, compared to some non-peptidic ligands) (e.g., Beyer et al., 2004) underlie its limited effectiveness *in vivo* in this model.

The centrally penetrating μ-agonist fentanyl produced robust dose-dependent prolactin release in all four subjects, as previously found for high efficacy μ-agonists in humans and non-human primates (Hoehe et al., 1988; Bowen et al.,

2002; Butelman et al., 2002). Interestingly, quaternary naltrexone (1 mg/kg) only partially blocked this effect of fentanyl, whereas this quaternary naltrexone dose fully blocked the effects of loperamide herein. It cannot be excluded that a larger dose of quaternary naltrexone could have produced full blockade of fentanyl in this setting (such a dose could not be studied, due to supply limitations). However, it is possible that in addition to hypothalamic sites functionally outside the blood–brain barrier, μ-agonists may also cause prolactin release in primates by acting at sites inside the blood–brain barrier (consistent with some findings in rodents) (Armstrong and Hatton, 1980; Panerai et al., 1981; Odio and Brodish, 1990; Merchenthaler, 1991). The observed full blockade of fentanyl's effect by nalmefene (which would be postulated to occupy μ-receptors both inside and outside the blood–brain barrier) is consistent with the latter interpretation (France and Gerak, 1994; Butelman et al., 2002).

Endogenous β-endorphin in the periphery is thought to act as a paracrine or endocrine signal; it is possible that opioid neuropeptides undergo *in vivo* biotransformation, yielding potentially active or inactive fragments. In view of i.v. β-endorphin's low effectiveness in this biomarker assay, we wanted to initially determine whether rapid biotransformation into inactive fragment(s) was occurring. Most available radioimmunoassay are not able to unequivocally distinguish many potential β-endorphin biotransformation fragments (for background, see (Schulz et al., 2006). Therefore, we optimized a MALDI-MS analytical approach to detect β-endorphin within the expected concentration range for these studies, and that would be able to unequivocally identify potential β-endorphin fragments. *Ex vivo*, β-endorphin displayed relative stability when incubated in blood at body temperature, even up to 60 min, suggesting that it is not highly labile under these conditions. Further, *in vivo* biotransformation studies with one of the larger β-endorphin doses used herein (0.1 mg/kg), resulted in detection of full-length β-endorphin for at least 5 min in each of the subjects. Prior studies suggest that the kidney may be a site of removal for β-endorphin from the circulation (Sato et al., 1987; Thornton and Losowsky, 1997), and there also appears to be a moderate amount of binding to serum protein (approximately 35%) (Sato et al., 1985). Absolute quantification of β-endorphin concentration was not possible within these MALDI-MS assays, but relative quantification could be obtained by normalization to an identified constitutive baseline plasma peptide. Given that robust neuroendocrine effects can be observed within 5 min of i.v. bolus administration in this assay (e.g., for loperamide and fentanyl), it appears that rapid biotransformation is not the only factor underlying β-endorphin's relative ineffectiveness herein.

In summary, the present studies constitute an initial systematic comparison of the effects of β-endorphin in a translationally viable neuroendocrine biomarker assay, with the peripherally selective μ-agonist loperamide, and the centrally penetrating μ-agonist fentanyl (both in clinical use). β-Endorphin's neuroendocrine effects in gonadally intact male non-human primates were smaller than those of either non-peptidic agonist, and also exhibited clear inter-subject variability. This relatively small effect of β-endorphin was not apparently due to low efficacy at μ-receptors, and was also not solely due to rapid biotransformation into inactive fragments, as determined by a modified MALDI-MS technique. These studies also determined that a μ-receptor population functionally outside the blood–brain barrier is able to mediate prolactin release in primates.

Role of the funding sources

The funding source did not have further direct role in the design and analysis of the studies, or in manuscript preparation.

Conflict of interest

The authors have no conflicts of interest of relevance to these studies.

Acknowledgments

The authors gratefully acknowledge funding by NIH grants DA17369 (ERB), DA00049 and DA05130 (MJK) and RR00862 (BTC).

The authors also gratefully acknowledge the technical expertise of Beatrix Ueberheide for tandem MS studies, and Matthew Randesi for genotyping.

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