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ORIGINAL INVESTIGATION

Induction of morphine-6-glucuronide synthesis by heroin self-administration in the rat

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Abstract
Rationale Heroin is rapidly metabolized to morphine that in turn is transformed into morphine-3-glucuronide (M3G), an inactive metabolite at mu-opioid receptor (MOR), and morphine-6-glucuronide (M6G), a potent MOR agonist. We have found that rats that had received repeated intraperitoneal injections of heroin exhibit measurable levels of M6G (which is usually undetectable in this species).
Objective The goal of the present study was to investigate whether M6G synthesis can be induced by intravenous (i.v.) heroin self-administration (SA).
Materials and methods Rats were trained to self-administer either heroin (50 μg/kg per infusion) or saline for 20 consecutive 6-h sessions and then challenged with an intraperitoneal challenge of 10 mg/kg of heroin. Plasma levels of heroin, morphine, 6-mono-acetyl morphine, M3G, and M6G were quantified 2 h after the challenge. In vitro morphine glucuronidation was studied in microsomal preparations obtained from the liver of the same rats.
Results Heroin SA induced the synthesis of M6G, as indicated by detectable plasma levels of M6G (89.7±37.0 ng/ml vs. 7.35±7.35 ng/ml after saline SA). Most important, the in vitro V_{max} for M6G synthesis was correlated with plasma levels of M6G (r^2=0.78). Microsomal preparations from saline SA rats produced negligible amounts of M6G.
Conclusion Both in vivo and in vitro data indicate that i.v. heroin SA induces the synthesis of M6G. These data are discussed in the light of previous studies conducted in heroin addicts indicating that in humans heroin enhances the synthesis of the active metabolite of heroin and morphine.

Keywords Drug addiction · Drug abuse · Opiates · Morphine-3-glucuronide · Morphine-6-glucuronide · M3G · M6G · Liver microsomes · Microsomal preparations

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Introduction

In humans and other mammals, heroin is rapidly transformed, after absorption, in 6-monoacetylmorphine (6-MAM), which is further deacetylated to morphine. The metabolism of morphine mainly consists of the glucuronidation to either morphine-3-glucuronide (M3G) or to morphine-6-glucuronide (M6G) (Milne et al. 1996). Heroin metabolites are widely thought to be responsible for the neuropsychopharmacological effects of the parent compound (Gutstein and Akil 2006).

Contrary to M3G, M6G is a potent agonist at mu-opioid peptide receptors (MORs) (Ulens et al. 2001; Penson et al. 2000; Christrup 1997), and there is some evidence that, like heroin, it acts at a MOR-1 splice variant that has little affinity for morphine (Pan et al. 2009). Although M6G is less lipophilic than the parent compound and does not easily cross the blood–brain barrier (Meineke et al. 2002), it

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does reach the central nervous system (CNS) and its CNS clearance is significantly lower than systemic clearance (Lotsch 2005; Tunblad et al. 2005). Furthermore, the distribution of M6G in the rat brain is mostly extracellular, suggesting that after morphine administration, M6G concentrations at MOR are not far from those of the parent compound (Stain-Texier et al. 1999). Indeed, there is now substantial evidence that M6G contributes to both the analgesic and toxic effects of morphine. Morphine-6-glucuronide has been shown, for example, to produce analgesia when administered systemically (Romberg et al. 2004, Skarke et al. 2003) and is now under development as a therapeutic agent (Binning et al. 2011). Furthermore, central nervous system-depressant effects produced by repeated morphine administrations in patients with renal failure have been attributed to increased high blood levels of M6G due to impaired excretion (Pauli-Magnus et al. 1999; Peterson et al. 1990). M6G may also be implicated in the well-known individual differences in the responsiveness to morphine. The unusual resistance to morphine overdosing exhibited by some nephropatic patients, for example, has been attributed to a single-nucleotide polymorphism of the MOR gene, resulting in reduced responsiveness to M6G but not to morphine (Lotsch et al. 2002).

There is also some evidence that M6G plays a role in heroin reward (Walker et al. 1999), and thus it is possible that this metabolite is implicated in the natural history of heroin addiction. We have previously found that plasma and urine of heroin addicts contain more M6G and less M3G than those of heroin-naive individuals treated with morphine for pain control (Antonilli et al. 2003a)—which is quite remarkable, given that morphine exposure even at high doses and for long periods of time does not appear to influence M3G or M6G synthesis (Faura et al. 1998; Vermeire et al. 1998; Andersen et al. 2004). This has led us to hypothesize that the increased synthesis of M6G may contribute to the vulnerability to heroin addiction. Of course, this possibility cannot be easily explored in human addicts and calls for the use of animal models.

Intravenous drug self-administration in the rat is widely considered as a robust animal model of drug taking (Markou et al. 1993). Rats are generally thought to produce no M6G (Milne et al. 1996). Yet, relatively small amounts of this metabolite have been detected in adult rats (Wang et al. 2005). Most important, we have shown that repeated non-contingent intraperitoneal (i.p.) injections of high doses of heroin (but not of morphine) can induce the synthesis M6G in the rat (Antonilli et al. 2003b, 2005). Furthermore, microsomal preparations obtained from the livers of heroin-treated rats yielded, when incubated with morphine, measurable quantities of M6G, which was not detectable in microsomal preparations from rats treated with saline (Antonilli et al. 2003b, 2005).

These preliminary findings suggest that the rat may represent a viable model of heroin abuse even with respect to M6G synthesis. The major aim of the present study was to verify this possibility by investigating in vivo and in vitro synthesis of M6G after intravenous (i.v.) heroin self-administration in the rat.

Materials and methods

Animals

The study was conducted using 15 male Sprague–Dawley rats (Harlan Italy, San Pietro al Natisone, Italy) weighing 275 g at their arrival in the laboratory. Notice that one additional rat was tested but was excluded from the analyses because of catheter occlusion. Throughout the experiment, the rats were housed and tested in a dedicated temperature-controlled and humidity-controlled room, with free access to food and water (except during the test sessions) under a 14-h dark/10-h light cycle (lights off at 0700 hours). After their arrival, the rats were housed two per cage for 7–10 days before the surgery. After the surgery, the rats were housed individually. All procedures were in accordance with the Italian Law on Animal Research (DLGS 116/92) and with the guidelines for the care and use of laboratory animals issued by the Italian Ministry of Health.

Surgery

The catheter consisted of 10.5 cm of silicone tubing (0.37-mm inner diameter, 0.94-mm outer diameter) sheathed at 3.4 cm from its proximal end by a 5-mm-long heat-shrink tubing. On the day of surgery, the rats received an i.p. injection of 2.33 mg of xylazine hydrochloride (Rompun®, Bayer HealthCare) and an intramuscular injection of 14,000 IU of benzylpenicillin (Fournier Pharma, S. Palomba, Italy). The rats were then anesthetized with an i.p. injection of 0.56 ml/kg of Zoletil 100® (Virbac, Carros, France), containing tiletamine (50 mg/ml) and zolazepam (50 mg/ml). By using standard surgical procedures, the catheter was inserted into the right jugular vein, so as to reach the right atrium with its proximal end, and was then secured to the surrounding soft tissues with silk thread. The distal end of the catheter was passed subcutaneously in front of the left shoulder, externalized through a small incision at the nape of the neck, and connected to an L-shaped 22-gauge cannula. The cannula was then secured to the rat's skull using dental cement and stainless steel screws. After surgery, the rats were given 15 mg i.v. enrofloxacin (Baytril®, KVP Pharma + Veterinär Produkte Gmbh, Kiel, Germany). Catheters were flushed daily (at

1800 hours) with 0.1 ml of a sterile saline solution containing 0.4 mg of enrofloxacin and 25 IU heparin (Marvecs Services, Agrate Brianza, Italy).

Apparatus

The apparatus consisted of SA chambers made of transparent plastic, aluminum, and stainless steel grid floor. Plastic trays covered with pinewood shaving were placed under the grid floors. Each chamber was equipped with two retractable levers, positioned on the left-hand wall 12.5 cm apart and 9 cm above the floor, with cue lights positioned above each lever and a counterbalanced arm holding a liquid swivel. The SA chambers were placed within sound-attenuating and light-attenuating cubicles. Each cage was connected via an electronic interface to a syringe pump (Razel Scientific Instruments, St. Albans, VT, USA) and to a programmable logic controller (PLC; Allen Bradley, Milwaukee, WI, USA), in turn connected to a PC. Chambers, accessories, and electronic interfaces were purchased from ESATEL S.r.l. (Rome, Italy), and custom-developed control software was from Aries Sistemi S.r.l. (Rome, Italy). The infusion line consisted of a length of silastic tubing protected by a stainless steel spring and connected (through the liquid swivel and another length of silastic tubing) to a syringe positioned on the pump (which was programmed to work at an infusion rate of 10 μl/s).

Procedures

After the surgery, the rats were housed in the SA chambers where they remained for the entire duration of the experiment, which consisted of 20 daily sessions. All test sessions lasted 6 h and took place during the dark phase, between 1000 and 1600 hours, 7 days a week. Testing began 1 week after the surgery. Before the start of each session, the syringe pumps were activated, so as to fill the infusion lines, which were then connected to the catheters. During the 60 s preceding the start of each SA session, food and water were removed from the cage. Self-administered drug infusions and primings consisted of 40 μl of drug solution (or vehicle) and were delivered over a period of 4 s. During the SA sessions, the doors of the cubicles were kept closed. At the start of each session, the two levers were extended and remained extended for the entire duration of the session (except during the time-out periods; see the next paragraph). Only one of the two levers was active: that is, it triggered upon completion of the task an infusion of 50 μg/kg of heroin, whereas the other lever had no direct consequences on heroin infusion. Eleven rats self-administered heroin whereas four rats self-administered saline.

The number of consecutive responses required to obtain on a fixed ratio (FR) schedule a single infusion was raised from FR1 (sessions 1–4) to FR2 (sessions 5–20). Upon completion of the task, both levers were retracted and extended again after 40 s (time-out). The three lights above the active lever were on when the lever was extended and off when the lever was retracted. No other light cue was provided. Pressing on the inactive lever produced no lever retraction but did reset the counter of the active lever. On the first test session, all animals were placed with their forepaws on the active lever (time 0 min), so as to trigger a priming infusion. Priming infusions were administered again at times 60 and 120 min to animals that had not spontaneously self-administered at least one infusion during time periods 0–60 and 60–120 min, respectively. On sessions 2–7, priming infusions were administered at times 5, 60, and 120 min to animals that had not spontaneously self-administered at least one infusion during time periods 0–5, 5–60, and 60–120 min, respectively. On average, the rats received 0.8 primings per session. No primings were administered on sessions 8–20. The rats were allowed to self-administer a maximum of 100 infusions of heroin per session to minimize the risk of overdosing.

The day after the last SA session, all rats received at 1400 hours a challenge of 10 mg/kg of heroin i.p. (as done in previous studies; Antonilli et al. 2003b, 2005) and after 2 h were sacrificed to obtain blood samples for the quantification of heroin, 6-monoacetylmorphine (6-MAM), morphine, M3G, and M6G (see "Microsomal preparations"), and their livers were excised to obtain microsomal preparations (see "Microsomal preparations").

Microsomal preparations

Liver microsomes were prepared as previously described (Antonilli et al. 2003b). Briefly, tissues were minced and rinsed in ice-cold 1.15% KCl and homogenized in three volumes of 100 mM phosphate buffer (pH 7.4) containing 0.25 M sucrose. The homogenate was centrifuged for 20 min at 9,000 × *g*. The supernatant was further centrifuged for 60 min at 105,000 × *g*. The resulting microsomal pellet was resuspended in 100 mM phosphate buffer containing 0.25 M sucrose.

Glucuronidation assays

The morphine glucuronidation assay was performed as described by Wielbo et al. (1993). Microsomal preparations were resuspended in 100 mM phosphate buffer (pH 7.4) to a final protein concentration of 1.0 mg/ml. Microsomes were preincubated for 20 min in 0.05% deoxycholic acid at 4°C to achieve full enzymatic activity. Morphine concentrations ranged from 0.1 to 4 mM for the calculation of M3G and M6G kinetics. The incubation mixture consisted of 2 mM UDP-glucuronic acid (UDPGA), 100 mM phosphate buffer (pH 7.4), microsomes, and morphine (as

substrate) to a final volume of 0.3 ml. The reaction was started adding UDPGA. Sample and blanks (without UDPGA) were incubated in triplicates at 37°C for 30 min. The reaction was stopped with 0.2 ml of ice-cold acetonitrile, and all samples were kept at 4°C for 15 min; then they were centrifuged for 10 min at 5,800×*g*.

Sample preparation

Supernatants of incubation and plasma samples underwent solid phase extraction on reversed-phase/strong cation-exchange sorbent Strata-X-C (96-well plates, 30 mg) (Phenomenex, Torrance, CA). Cartridges were conditioned with methanol (0.6 ml) followed by water (0.6 ml) and phosphate buffer (0.01 M pH 3.0, 0.6 ml). The sample (0.1 ml) was applied to the column and absorbed by gravity; then the column was washed with phosphate buffer (0.01 M pH 3.0, 0.6 ml) and dried for 30 s. The analytes were eluted with 0.2 ml of NH_4OH 1% in methanol. The eluate was evaporated to dryness at 37°C under a nitrogen stream. The residue was dissolved in 0.2 ml of 5 mM ammonium formate buffer (pH 4.0) and stored at 4°C until LC/MS/MS analysis.

Liquid chromatography and mass spectrometry

The HPLC system consisted of a PerkinElmer 200 Series binary pump and autosampler (PerkinElmer, Norwalk, CT, USA) and an SCIEX API2000MS/MS triple quadrupole mass spectrometer (Applied Biosystem-MDS SCIEX, Thornhill, Ontario, Canada). Incubation and plasma samples were injected onto a LiChroCART® Purospher Star RP-18 column (150×4.6 mm i.d., particle size 5 μm) with a LiChroCART® Purospher Star RP-18 precolumn (4×4 mm, particle size 5 μm; Merck). The mobile phase consisted of a linear gradient (3–80% with respect to acetonitrile) formed by combination of 5 mM ammonium formate buffer in water (pH 4.0, eluent A) and acetonitrile (eluent B). Flow rate of the mobile phase was set at 0.8 ml/min. Heroin, 6-MAM, morphine, M3G, and M6G were detected using multiple reaction monitoring (MRM) in positive ionization mode. Selected ion masses of the protonated precursors and fragmented ions (*m*/*z*) were 370.1/268.0, 328.1/165.0, 286.3/201.0, and 462.2/286.0 for heroin, 6-MAM, morphine, M3G, and M6G respectively. Chromatographic peaks were integrated using Analyst™ software (version 1.4.1, SCIEX). The detection limits (LOD) and quantification limits (LOQ) for all analytes were 5 and 10 ng/ml, respectively.

Statistical analyses

Plasma levels of heroin, morphine, 6-MAM, M3G, and M6G were analyzed using two-tailed Student *t*-tests. Furthermore, M6G data were also analyzed using the Fisher exact probability test, by classifying the rats as M6G synthesizers versus non-M6G synthesizers (i.e., rats with undetectable plasma levels of M6G).

The saturation curves for the formation of M3G and M6G by liver microsomes leveled off at the highest morphine concentrations. K_m (mM), V_{max} (nmol/min/mg protein), and Hill coefficient of M3G and M6G formation were estimated using nonlinear regression analysis (GraphPad Prism 3; GraphPad Software Inc., San Diego, CA, USA).

A Hill coefficient greater than 1 indicates that an enzymatic reaction does not follow Michaelis–Menten kinetics; that is, there is positive cooperation in the catalytic activity. In the presence of data satisfying the normality test, group differences for K_m, V_{max}, and Hill coefficient were investigated using one-way ANOVAs. When appropriate, Fisher post hoc test was used for pairwise comparisons. The K_m and V_{max} values in Exp. 2 were analyzed using nonparametric statistics (Kruskal–Wallis ANOVA and Dunn's pairwise multiple comparison procedure) because these data failed the normality test (p=0.004 and p=0.004, respectively).

Results

Figure 1 illustrates the number of lever presses on the active vs. the inactive lever for rats self-administering heroin or saline. During the 20 sessions, the rats self-administered a total amount of 18.26±1.88 mg/kg of heroin.

Table 1 illustrates the plasma levels of heroin, morphine, 6-MAM, M3G, and M6G in rats that had self-administered heroin vs. saline. As predicted, rats that had self-administered heroin exhibited sizeable plasma levels of M6G, in contrast to the negligible levels seen in rats that had self-administered saline (p=0.052). Indeed, 91% of heroin rats exhibited detectable levels of M6G versus 25% of saline rats (Fisher exact probability test, p=0.033). Plasma levels of M3G were about 50% greater in the heroin SA group than in the saline SA group, but this difference was not significant (p=0.34).

Figure 2 and Table 2 illustrate the kinetics of in vivo M3G and M6G synthesis when hepatic microsomal preparations were incubated with morphine. Consistent with the in vivo data, negligible amounts of M6G were synthesized in vitro by the microsomal preparations obtained from rats that had self-administered saline (V_{max} and K_m could be calculated only in one rat). In contrast, a significant amount of M6G was synthesized by the microsomal preparations from rats that had self-administered heroin. As illustrated in Fig. 3, the synthesis of M6G appeared to be the result of positive enzymatic

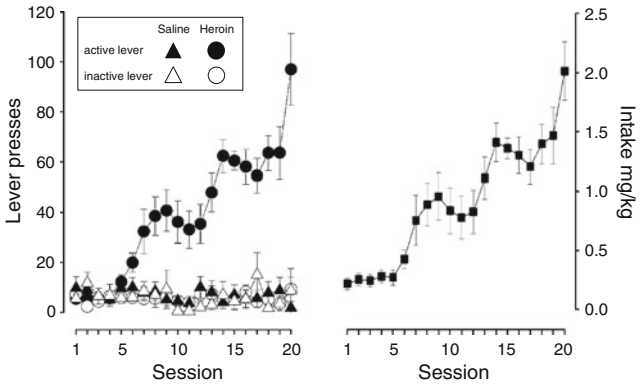

Fig. 1 Number of presses on the active vs. inactive lever (means ± SEM) for rats self-administering saline or heroin (50 μg/kg) on an FR1 (sessions 1–4) and then FR2 (sessions 5–20) schedule of reinforcement

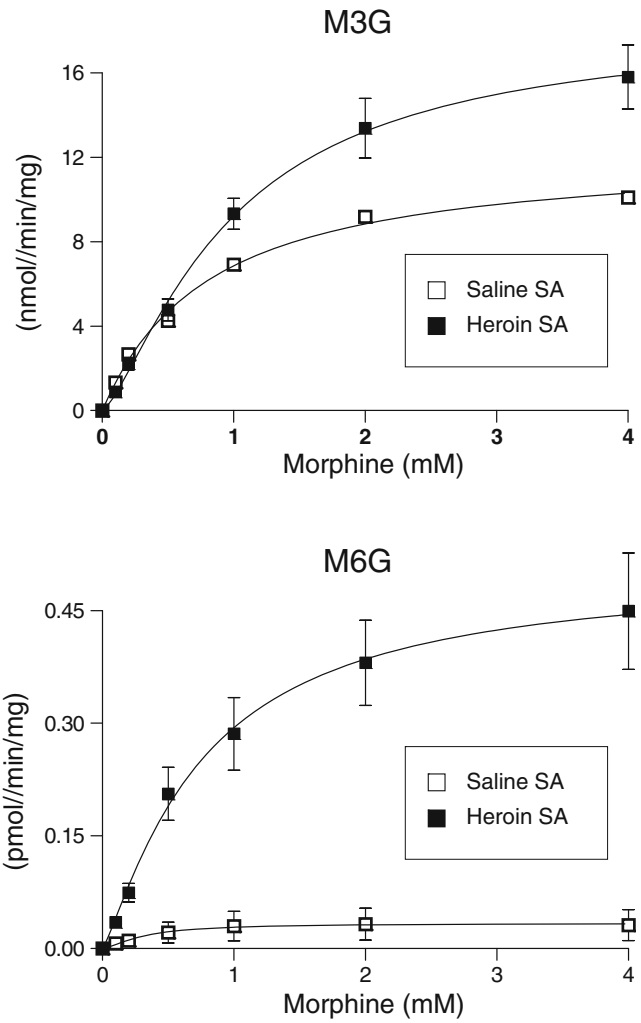

Fig. 2 Kinetic of M3G and M6G formation by microsomal preparations obtained from the liver of rats treated with saline or heroin and incubated with increasing concentrations of morphine. Each data point is an average of triplicate determination ± SEM

cooperation (Hill coefficient = 1.90±0.34). Most important, the in vitro V_{max} for M6G synthesis was correlated with plasma levels of M6G (r^2=0.78, p<0.001) (Fig. 3) and with the amount of heroin self-administered during training (r^2=0.41, p=0.01). Thus, it is not surprising that there was also a significant correlation between M6G levels and the amount of heroin self-administered during training (r^2=0.31, p=0.035).

As illustrated in Fig. 4, the curve of M3G formation in the microsomal preparation obtained from saline SA rats was in agreement with standard Michaelis–Menten kinetics (Hill coefficient = 1.00±0.09) whereas positive enzymatic cooperation was evident in the case of rats that had self-administered heroin (Hill coefficient = 1.40±0.17; p=0.053 vs. saline). Positive enzymatic cooperation for M3G synthesis was independent of positive enzymatic cooperation for M6G synthesis, as indicated by the lack of correlation between the respective Hill coefficients (r^2= 0.03, p=0.63). The in vitro V_{max} of M3G synthesis was about 50% greater in the heroin SA group than in the saline SA group (p=0.022), but there was no correlation between the V_{max} of M3G synthesis and plasma levels of M3G. The in vitro K_m of M3G synthesis was also greater in the heroin SA group than in the saline SA group, but this difference only approached significance (p=0.056).

Discussion

In the present study, we investigated the synthesis of M6G, an active metabolite of heroin and morphine and a powerful MOR agonist, in a rat model of heroin abuse. We found that

Table 1 Mean (± SEM) plasma levels (nanograms per milliliter) of heroin, 6-MAM, morphine, M3G, and M6G (ad their sum) in samples obtained 2 h after a single i.p. injection of 10 mg/kg of heroin, administered the day after the last of 20 sessions of heroin or saline self-administration

	Heroin	6-MAM	Morphine	M3G	M6G*	Total
Saline	17.00±17.00	170.72±115.11	121.42±35.99	330.92±167.46	7.35±7.35	646.38±158.99
Heroin	9.03±9.03	46.81±29.37	178.25±80.92	534.82±97.89	90.00±36.84	858.77±126.38

*p<0.05 vs. saline

Table 2 Kinetics of morphine glucuronidation in microsomal preparation obtained from livers excised 2 h after a single i.p. injection of 10 mg/kg of heroin, administered the day after the last of 20 sessions of heroin or saline self-administration (same rats of Table 1)

	M3G				M6G			
	K_m (mM)	V_{max} (nmol/min/mg)	V_{max}/K_m	Hill coeff.	K_m (mM)	V_{max} (pmol/min/mg)	V_{max}/K_m	Hill coeff.
Saline	0.82±0.16	12.11±1.32	16.48±3.50	1.00±0.09	0.31[a]	0.13[a]	0.42[a]	1.5[a]
Heroin	0.97±0.21	18.31±2.07*	21.30±2.04	1.40±0.17*	0.57±0.16	0.39±0.12	0.79±0.14	1.55±0.36

Data are expressed as means ± SEM

coeff. coefficient

*$p<0.05$ vs. saline

[a] V_{max} and K_m of M6G synthesis could be calculated only in one saline rat

heroin SA powerfully induced the synthesis of M6G both in vivo (as indicated by detectable plasma levels) and in vitro (in microsomal preparations, obtained from the rats' livers, incubated with morphine).

These findings appear to be at odds with the notion that rats produce no M6G (Milne et al. 1996). However, we have previously shown that M6G can be induced by repeated non-contingent i.p. administrations of heroin (Antonilli et al. 2003b, 2005), and there is evidence that adult rats can synthesize M6G even under basal conditions (Wang et al. 2005). Microsomal preparations, obtained from the livers of these rats, yielded, when incubated with morphine, significant concentration of M6G (which was absent in the microsomal preparations obtained from saline-treated rats). However, in these earlier studies the heroin pretreatment consisted of high i.p. doses of heroin (10 mg/kg×10). Here we show that M6G is formed in even larger amounts in rats self-administering heroin i.v. These elevated plasma levels of M6G were clearly the result of increased synthesis, as indicated by the correlation between plasma levels of M6G and microsomal M6G synthesis in vitro. This conclusion is further supported by the results of other in vitro experiments with isolated rat hepatocytes. When hepatocyte cultures were pre-incubated for 72 h with heroin and then incubated with morphine, significant amounts of M6G were produced, as opposed to cultures pre-incubated with vehicle (Graziani et al. 2008).

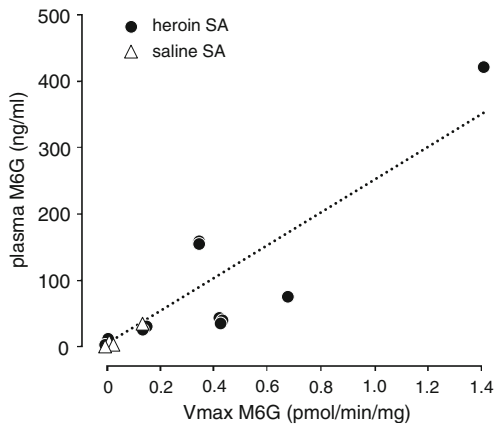

Fig. 3 Regression of plasma levels of M6G over in vitro V_{max} of M6G formation by liver microsomes

The results obtained with heroin SA do not completely overlap with those obtained with non-contingent i.p. administrations of heroin. In particular, there were two major discrepancies. First, in vitro synthesis of M3G was reduced after repeated i.p. injections of heroin (Antonilli et al. 2005; Graziani et al. 2008) but not after heroin SA. Second, M3G and M6G synthesis followed standard Michaelis–Menten kinetics (Hill coefficient = 1) in microsomal preparations obtained from the rats that had received repeated i.p injections of heroin (Antonilli et al. 2005) but not in those obtained from the rats that had self-administered heroin. The reasons of these discrepancies are not clear, as there are many differences in drug regimen between the two procedures (e.g., route of administration, self-administration vs. non-contingent administration, drug amount, etc.).

The mechanisms responsible for the ability of heroin SA to modulate morphine glucuronidation are not known. These effects were not mimicked by methadone nor blocked by naltrexone, suggesting MOR-independent mechanism(s) of action (Antonilli et al. 2005; Graziani et al. 2008). Furthermore, the fact that similar results were obtained with liver microsomes (Antonilli et al. 2005) and isolated hepatocytes (Graziani et al. 2008) indicates that heroin can alter morphine glucuronidation by acting directly on the liver. Interestingly, we found here that the variability in the V_{max} of M6G synthesis by liver microsomes accounted for about 80% of the variance in plasma levels of M6G. Finally, Hill coefficients greater than 1 for the synthesis of M3G and M6G indicate enzymatic cooperativity. Taken together these data suggest that heroin acted at a post-translational level by inducing homodimerization or heterodimerization of UGTs. This hypothesis requires further investigation.

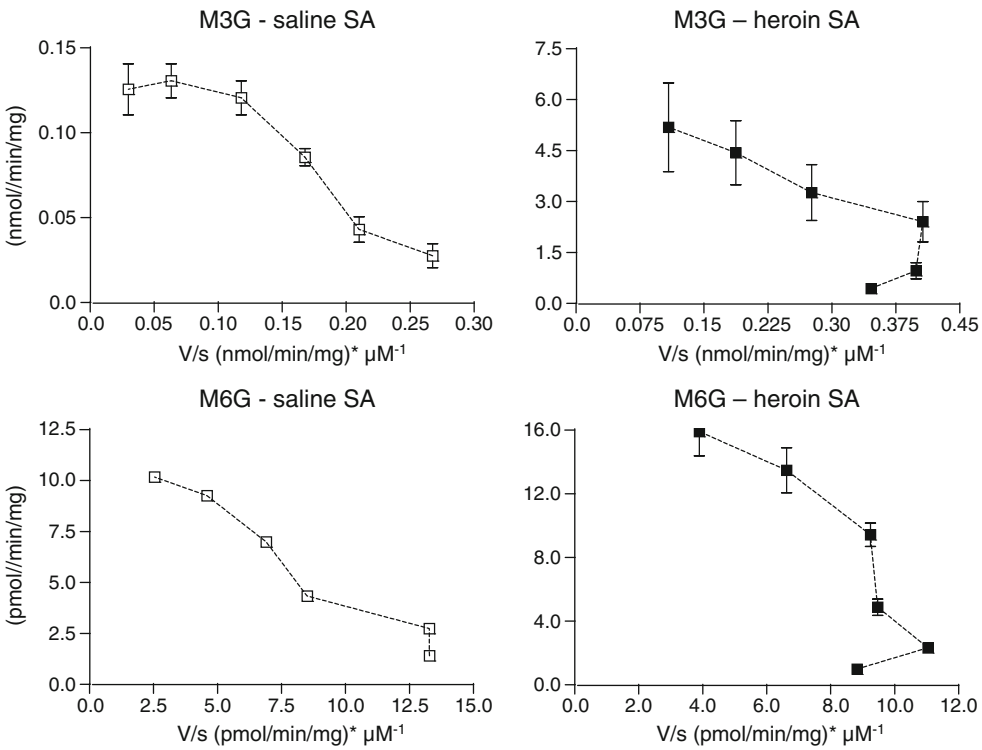

Fig. 4 Eadie–Hofstee plots of M3G and M6G formation by microsomal preparations from rats that had self-administered saline or heroin. From the same data of Fig. 2

In addition, it is possible that the effects of heroin exposure on morphine glucuronidation depended on changes in the expression of genes encoding for UGTs. We have recently found that 72-h exposure of rat hepatocytes to heroin reduces the expression of both UGT1A1 and UGT1A6 genes, whereas the expression of the UGT2B1 gene was significantly enhanced (unpublished data). It is not yet clear how heroin elicits these changes in the expression of UGTs genes. The most plausible targets of heroin action are the ligand-activated transcription factors that regulate the expression of a wide array of enzymes involved in detoxification, including UGTs. Although there is no direct evidence of an action of heroin on these transcriptional factors, nuclear opioid binding sites associated with regulatory protein kinase C have been identified by Ventura et al. (2003) in cardiac cells. More recently, it has been found that morphine enhances the expression of TNFα in astrocytes and microglia by facilitating translocation of the NF-κB class of transcription factors from the cytoplasm to the nucleus (Sawaya et al. 2009). More experiments are then required to explore the possibility that heroin modulates UGTs expression in the liver by interacting with nuclear transcriptional factors.

The results reported here show that intravenous heroin self-administration can induce M6G synthesis even in the rat. This suggests that the increase in the plasma M6G concentration previously observed in human addicts (Antonilli et al. 2003a) was not a mere epiphenomenon in the natural history of heroin addiction. What are the possible implications of this finding?

Morphine-6-glucuronide does not easily cross the blood–brain barrier (Meineke et al. 2002), but its distribution in the brain is mostly extracellular, suggesting that its concentrations at MOR are not far from those of parent compounds (Stain-Texier et al. 1999). After intracerebroventricular or intrathecal injection, M6G has been reported to be one to two orders of magnitude more potent than morphine, with respect to its analgesic and ventilatory effects (Paul et al. 1989; Gong et al. 1991; Frances et al. 1992). The greater potency of M6G has been attributed to greater efficacy in activating the MOR (Osborne et al. 2000; Ulens et al. 2001) or to its actions at a unique MOR subtype. The existence of a MOR-1 subtype with greater affinity for M6G than for morphine was first proposed by Rossi et al. (1995a) and was later confirmed by others (Brown et al. 1997; Mantione et al. 2002). Experiments using antisense probes or knockout mice have demonstrated the existence of splice variants of MOR-1 with differential affinity for morphine versus heroin and M6G (Rossi et al. 1995b; Matthes et al. 1996; Sora et al. 1997; Loh et al. 1998; Schuller et al. 1999; Unterwald et al. 1999; Pan et al. 2009). In addition to being a potent MOR agonist, M6G exhibits a much longer half-life than heroin or morphine. The delay between peak plasma concentrations and

analgesic effects in humans, for example, is 2–3 h for morphine versus 7 h for M6G (Lotsch 2005). Hence, the effects of M6G may largely outlast those produced by the parent compounds. In particular, having the same pharmacological profile of heroin, M6G may significantly contribute to the short-lived reinforcing effects of the former, which have been long known to differ from those of morphine (Fraser et al. 1961; Martin and Fraser 1961). Pharmacological antagonism of M6G has been shown to block heroin self-administration (Walker et al. 1999). It follows that all conditions leading to increased synthesis of M6G might play a role in the development of heroin addiction.

In conclusion, the present findings may have important implications for the study of heroin addiction in humans. The exact relationship between the changes in M6G synthesis and the development of addiction, if any, remains to be determined. For example, it is possible that the induction of M6G synthesis represents a mere consequence of repeated exposure to heroin. We are now conducting experiments to investigate the existence of a causal relationship between individual variability in the ability to synthesize M6G and the propensity to develop heroin addiction.

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