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In vivo chronic exposure to heroin or naltrexone selectively inhibits liver microsome formation of estradiol-3-glucuronide in the rat

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ARTICLE INFO

Article history:
Received 23 April 2008
Accepted 12 June 2008

Keywords:
Heroin
Naltrexone
Estradiol-3-glucuronide
Estradiol-17-glucuronide
UGT1A1

ABSTRACT

We have previously found that repeated exposure to heroin reduces liver synthesis of morphine-3-glucuronide (M3G) and increases the production of morphine-6-glucuronide (M6G), which normally is not formed in the rat. By contrast repeated exposure to naltrexone does not activate M6G synthesis but increases the V_{max} of M3G formation. M3G synthesis depends on the activity of two isoforms of the UDP-glucuronosyltransferase (UGT), UGT1A1 and UGT2B1. These isozymes also activate the formation of estradiol-3-glucuronide (E3G) and estradiol-17-glucuronide (E17G), respectively. The goal of the present study was to investigate the role of UGT1A1 and UGT2B1 in the effects of heroin and naltrexone by determining their influence on the synthesis of E3G and E17G. Estradiol glucuronidation was performed using microsomes of rats treated daily, for 10 days, with saline, heroin (10 mg/kg, i.p.), or naltrexone (40 mg/kg, i.p.). Moreover, liver expression of both UGT1A1 and UGT2B1 was studied in the same experimental conditions by polymerase chain reaction analysis. Kinetic analysis showed that the V_{max} for E3G formation was significantly reduced by both heroin (168.82 ± 9.73 nmol/mg/min) and naltrexone (194.60 ± 16.6) relative to saline (624.60 ± 17.6). Moreover, homotropic kinetic of E3G formation (Hill coefficient: 1.8) was transformed in Michaelis–Menten kinetic by both heroin (0.88) and naltrexone (1.15). The synthesis of E17G was not affected by either opioid. The expression of liver UGT1A1 and UGT2B1 did not differ across groups. The present results suggest that heroin and naltrexone can reduce estradiol glucuronidation via a specific interaction with UGT1A1 isoform.

1. Introduction

UDP-glucuronosyltransferases (UGT) is a large family of isozymes responsible for glucuronidation, an enzymatic reaction that makes several endogenous and exogenous compounds available for biliar or renal excretion [1,2]. Typically, when two hydroxyl moieties of the same molecule are exposed to glucuronidation, one of the moieties is preferentially conjugated. In the case of morphine, for instance, formation of morphine-3-glucuronide (M3G) largely prevails on that of morphine-6-glucuronide (M6G) [1,3]. The interest in understanding the mechanism governing preferential morphine glucuronidation derives from the contrasting pharmacological effects of the two glucuronil-derivatives. In particular, M6G significantly contributes to morphine analgesic and hypnotic effects [4–6]. M3G, instead, seems to

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0006-2952/$ – see front matter
doi:10.1016/j.bcp.2008.06.011

be involved in the development of hyperalgesia induced by prolonged morphine administration [7,8]. Thus, changes in the relative rates of M3G and M6G formation may influence the overall effects induced by the administration of the parent compound. Although morphine administration has been found to generate plasma M3G/M6G ratios that are stable across a wide range of clinical settings [2], we observed that subjects chronically exposed to street heroin exhibit significantly lower M3G/M6G ratios with respect to opioid-naïve patients injected with morphine [9]. We found similar changes in morphine glucuronidation in ex vivo studies in which rats were repeatedly administered heroin [10,11]. These studies were conducted in microsomal preparation obtained from livers excised 2 h after the last drug treatment and then incubated with morphine. The fact that repeated administrations of heroin in the rat can induce the formation of M6G is quite remarkable, given that, under normal conditions, M6G is not synthesized in this species [1]. The pharmacological specificity of these effects is indicated by the fact that they were dose-related and were reversible upon discontinuation of heroin administration. However, the ability of heroin to reduce M3G synthesis while inducing that of M6G do not appear to depend on the activation of MOR, because these effects of heroin were not mimicked by methadone nor were they blocked by naltrexone. Furthermore, when administered alone, naltrexone exhibited its own effects on morphine metabolism, increasing the V_{max} and reducing the K_d of M3G synthesis. We recently found that the ex vivo effects of heroin on morphine glucuronidation can be reproduced *in vitro*. Primary cultures of rat hepatocytes pre-incubated with heroin and then incubated with morphine yielded less M3G than control cultures, as well as significant amounts of M6G, which was absent in control conditions [12].

The goal of the present study was to further explore the kinetics of glucuronidation under conditions of prolonged exposure to heroin or naltrexone. In the rat, at least two UGTs (UGT1A1 and UGT2B1) participate to morphine glucuronidation [13,14]. In an attempt to determine the relative contribution of UGT1A1 and UGT2B1 to the effects of heroin and naltrexone on morphine glucuronidation described above, we investigated here the effects of these drugs on the glucuronidation of estradiol. As in the case of morphine, the presence of two hydroxyl groups allows for the transformation of estradiol into two glucuronides: estradiol-3-glucuronide (E3G) and estradiol-17-glucuronide (E17G). The synthesis of E3G is catalyzed by UGT1A1 [15,16] while that of E17G is catalyzed by UGT2B1/3 [17,18]. The kinetics of estradiol glucuronidation was studied in microsomal preparations obtained from the livers of rats repeatedly exposed to heroin or naltrexone. In addition, we determined the hepatic levels of UGT1A1 and UGT2B1 mRNA in the liver of heroin- or naltrexone-treated rats was determined by reverse transcriptase polymerase chain reaction (PCR) analysis.

2. Materials and methods

2.1. Chemicals

Heroin hydrochloride was obtained from Salars (Como, Italy) and naltrexone hydrochloride from Sirton spa (Villaguardia, Como, Italy). Estradiol, estradiol-3-glucuronide, estradiol-17-glucuronide, uridinediphosphoglucuronic acid (UDPGA), alamethicin were obtained from Sigma–Aldrich (Milan, Italy). All solvents were HPLC grade (Merck, Darmstadt, Germany).

2.2. Animals

A total of 44 male Sprague–Dawley rats (Harlan Italy, S. Pietro al Natisone, Italy), weighing 150–200 g upon arrival and of approximately 6–8 weeks of age, were used in this study. Rats were individually housed in transparent plastic cages at 23 °C under a 12-h light/12-h dark cycle (lights on at 7:00 am). Animals had ad libitum access to food and water. All drug treatments were administered by i.p. injection at 12:00 am.

2.3. Experiment 1

Thirty rats were randomly distributed into three groups ($n = 10$). On days 1–10, rats received daily i.p. injections of 1 ml/kg of saline (0.9% NaCl), heroin (10 mg/kg) or naltrexone (40 mg/kg). Two hours after the last injection, animals were sacrificed by decapitation and livers excised.

2.4. Microsomal preparations

Liver microsomes were prepared as previously described [10]. Briefly, tissues were minced and rinsed in ice-cold 1.15% KCl and homogenized in 3 volumes of 100 mM phosphate buffer (pH 7.4) containing 0.25 M sucrose. The homogenate was centrifuged for 20 min at $9000 \times g$. The supernatant was further centrifuged for 60 min at $105{,}000 \times g$. The resulting microsomal pellet was resuspended in 100 mM phosphate buffer containing 0.25 M sucrose, to obtain a final protein concentration of 10 mg/ml.

2.5. Estradiol glucuronidation assay

The estradiol glucuronidation assay was performed as described by Alkharfy and Frye [19]. Tubes containing liver microsomes (1mg/ml final protein concentration) and alamethicin (30 μg/protein) were mixed and placed in ice for 15 min. The incubation mixture contained 100 mM potassium phosphate buffer (pH 7.4), 5 mM $MgCl_2$, estradiol (from 1 to 150 μM concentration range) and 5 mM UDPGA in a total volume of 300 μl. The reaction was started by adding UDPGA. Samples and blanks (without UDPGA) were incubated in triplicates at 37 °C for 30 min and the reaction was stopped by adding 10% perchloric acid (50 μl) and centrifuged. Supernatants were then submitted to HPLC analysis.

2.6. HPLC analysis

The HPLC system consisted of an automatic sampler (Model L-7250), pump (Model L-7100), diode array detector (Model L-7455), and fluorescence detector (Model L-7480), all purchased from Merck KGaA (Darmstadt, Germany). Data were stored and processed using appropriate software (D-7000 HPLC System Manager Vers. 3.1 Hitachi).

Separation was carried out on an Alltima phenyl column, 5 μm, 4.6 mm × 250 mm (ALLtech Associates). The mobile phase consisted of 35% acetonitrile and 65% 50 mM ammonium phosphate buffer (pH 3). The flow rate was set at 1 ml/min and the injection volume was 40 μl. Estradiol, E3G and E17G were quantified by fluorescence detection (excitation wavelength = 210 nm, emission wavelength = 350 nm) and UV diode array detection at 210 nm. LOQ was 60 pmol/ml for both E3G and E17G. Standard curve correlation coefficient (r^2) was ≥0.99.

2.7. Enzyme kinetic analysis

The apparent kinetic parameters of K_m, V_{max} and n were calculated by non-linear regression analysis (GraphPad Prism 3; GraphPad Software Inc., San Diego, CA, USA). The conventional Michaelis–Menten equation, or the Hill equation, were fit to the data. The quality of fit to a particular model was assessed evaluating the following criteria: (1) visual inspection of the Eadie-Hofste plot of the data; (2) the sum of the squares of the residuals; (3) the S.E. of the parameters estimated. Statistical analyses of group differences for rate values were conducted using one-way ANOVA. When appropriate, Tukey's tests were used for pair-wise comparisons. Significance level was set at $p < 0.05$.

2.8. Experiment 2

Fourteen male Sprague–Dawley rats were used in this experiment, which was aimed to determine the expression of both UGT1A1 and UGT2B1 genes in rat liver upon repeated exposure to saline, heroin or naltrexone. Following the first week of habituation, animals were randomly assigned to three treatment groups. On day 1–10, rats in the first group received a daily i.p. injection of saline 1 ml/kg ($n = 3$), while the other two groups received either heroin 10 mg/kg ($n = 7$) or naltrexone 40 mg/kg ($n = 4$). Two rats in the heroin group died during the treatment schedule, and their livers were not collected. On day 10, 2 h after the injection, rats were sacrificed by decapitation and their livers were excised and submitted to PCR analysis.

2.9. Polymerase chain reaction analysis

PCR analysis was performed as already described [20]. Samples derived from rat livers (approximately 40–60 mg in weight) were homogenized with an Ultra Turrax T8 (IKA Labortechnik, Staufen, Germany) homogenizer, then total RNA was extracted by using Absolutely RNA Miniprep kit (Stratagene, La Jolla, CA, USA) including DNaseI treatment, according to the manufacturer's instructions. The cDNA was synthesized from 2 μg of liver RNA, using ThermoScript reverse transcriptase (Invitrogen, Carlsbad, CA, USA) primed with random examers. Semi-quantitative RT-PCR was performed in a 25 μl reaction mixture using 2 ml of cDNA, 1.5 mM $MgCl_2$ and 0.3 ml of recombinant Taq DNA Polymerase (New England BioLabs). Primer pairs (0, 2 mM), selected according to NCBI mRNA sequences, were designed to hybridize only with rat UGT1A1 (GI: 89276773) and UGT2B1 (GI: 207580); as a positive control and for an internal standard we used the housekeeping gene b Actin (GI: 42475962). For negative controls, amplifications were performed using water as PCR template or omitting the reverse transcriptase step in the first strand synthesis reaction. Sequence of primers used for the amplification were the followings: UGT1A1: acaccggaactagaccatcg (forward)/ttggaaccccattgcatatt (reverse) product size 153 bp; UGT2B1: cgcatgacatttgtggaaag (forward)/gtcccagaaggttcgaatga (reverse), product size 180 bp; bACTIN: agtacaaccttcttgcagctcctc (forward)/ccatatcgtcccagttggtgac (reverse), product size 303 bp. RT-PCR products were analysed by 2% agarose gel stained with ethidium bromide and quantified using Sigma Scan Pro 5 software.

3. Results

3.1. Experiment 1

Fig. 1 and Table 1 illustrate kinetics of E3G and E17G formation from microsomal preparations obtained from the livers of rats repeatedly treated with saline, heroin or naltrexone. At visual inspection, the curve of E3G formation obtained from saline-

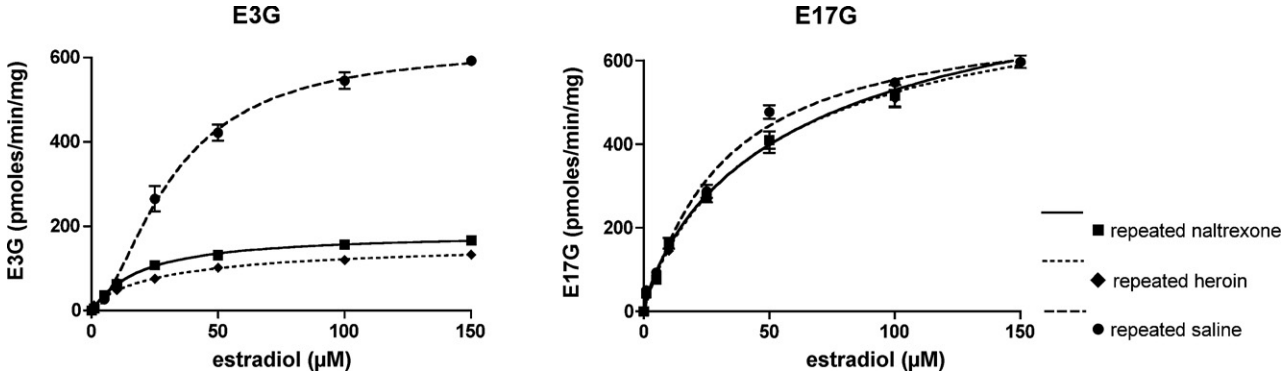

Fig. 1 – Kinetic of E3G (right panel) and E17G (left panel) formation by microsomal preparations obtained from the liver of rats treated with heroin or naltrexone and incubated with increasing concentrations of estradiol. Both heroin and naltrexone produced a remarkable reduction in the rate of synthesis of E3G, but not of E17G. In addition, the exposure to heroin or naltrexone transformed the curve of E3G synthesis from sigmoid to exponential. Each data point is an average of triplicate determinations ± S.E.M.

Table 1 – Kinetics of E3G and E17G formation by liver microsomes incubated with estradiol

	E3G			E17G		
	K_m (μM)	V_{max} (pmol/min/mg)	Hill constant	K_m (μM)	V_{max} (pmol/min/mg)	Hill constant
Repeated saline ($n = 10$)	32.71 ± 3.37	624.60 ± 17.6	1.69 ± 0.07	33.15 ± 2.49	724.00 ± 21.03	1.06 ± 0.07
Repeated heroin (10 mg/kg × 10) ($n = 10$)	31.66 ± 3.29	168.82 ± 9.73*	0.84 ± 0.03*	44.26 ± 8.73	802.90 ± 46.76	0.97 ± 0.05
Repeated naltrexone (40 mg/kg × 10) ($n = 10$)	26.43 ± 8.27	194.60 ± 16.6*	1.13 ± 0.07*	46.60 ± 12.57	858.4 ± 42.88*	0.89 ± 0.08

Microsomal preparations obtained from the liver of rats treated with saline, heroin and naltrexone were incubated with estradiol for 1 h.
* $p < 0.05$ vs saline.

injected rats showed the expected sigmoid shape. Hill equation confirmed that the formation of E3G did not follow Michaelis–Menten kinetic, since it yielded a mean value of 1.8 compatible with homotropic activation of the enzymatic activity [16]. Positive cooperation in the E3G formation was confirmed by the Eadie-Hofstee plot that showed the ''hooked curve'' typical of homotropic activation (Fig. 2A). Repeated administration of heroin or naltrexone changed the kinetic of E3G formation, but in different ways. Both treatments shifted the Hill coefficient to values close to the unit ($F_{2,28} = 26.38$, $P < 0.001$) and reduced the V_{max} of E3G formation ($F_{2,28} = 22.32$, $P < 0.001$) without affecting its K_m. Moreover, the Eadie-Hofstee plot showed that exposure to heroin transformed the ''hooked curve'' in the exponential relationship typical of Michaelis–Menten kinetic (Fig. 2B). In contrast, the hooked shape of Eadie-Hofstee plot was maintained in the naltrexone-treated group, although the flex point was shifted to a higher substrate concentration (Fig. 2C).

As expected, in the control group the formation of E17G followed Michaelis–Menten kinetics as shown by visual inspection of the saturation curve as well as by the Hill equation, which yielded a coefficient value very close to 1.0 (Fig. 1B and Table 1). Heroin treatment did not affect E17G formation whereas naltrexone only caused a small increase of V_{max}.

3.2. *Experiment 2*

Formation of E3G and E17G is mediated by UGT1A1 and UGT1B1, respectively. Considering that drug treatment affected the kinetics of E3G, but not of E17G formation, we considered the possibility that heroin and naltrexone alter the synthesis of UGT1A1, but not of UGT1B1 isozyme. However, as shown in Figs. 3 and 4, repeated exposure to heroin (10 mg/kg, i.p.) or to naltrexone (40 mg/kg, i.p.) did not change the expression of UGT1A1 (one-way ANOVA: $F_{2,11} = 1.545$, $P = 0.0265$) and UGT2B1 mRNA (one-way ANOVA: $F_{2,11} = 0.643$, $P = 0.7164$).

4. Discussion

The main finding reported in the present study is that repeated administrations of heroin or naltrexone to rats can alter estradiol glucuronidation in microsomal preparations obtained from livers excised 2 h after the last treatment. Using similar experimental procedures we previously found the repeated heroin and repeated naltrexone can alter morphine glucuronidation [10,11].

The fact that both heroin and naltrexone modified the kinetics of E3G formation leaving unchanged the formation of E17G is consistent with the notion that the two glucuronides are the product of separate enzymatic processes. The formation of E17G mainly depends on UGT2B1/3 activity [17,18], whereas E3G is the result of UGT1A1 activity [21,22]. Moreover, E17G formation depends on monomeric enzymatic activity as shown by an exponential reaction curve that is fully described by the Michaelis–Menten equation. In contrast, the curve of E3G formation shows a point of flex and this sigmoidicity is considered the result of homotropic UGT1A1 activation, which depends on the multimeric structure of this enzyme. The binding of a molecule of substrate to a subunit produces an increase in the affinity of a neighboring subunit for the substrate [15–17,23,24]. The Hill equation and the Eadie-Hofstee plot are useful mathematical tools for describing homotropic activations. In microsomal preparations obtained from control animals, the Hill coefficient value for E3G formation was on average 1.8, a value in line with that obtained in previous studies [16,25]. Moreover, the Eadie-Hofstee plot showed the hooked shape characteristic of homotropic activation. Repeated administrations of either heroin or naltrexone profoundly affected this kinetic, bringing the Hill coefficient to values close to the unit and reducing the V_{max} of E3G formation. The Eadie-Hofstee plot of E3G formation shows that the mechanisms of action of the two drugs are not identical, since in the case of naltrexone the plot maintained at least in part the hooked shape whereas in the case of heroin there was a complete transformation of the plot indicating with the acquisition of Michaelis–Menten kinetics. Thus, heroin was much more effective than naltrexone in preventing the homotropic activation of UGT1A1. It has been demonstrated that the homotropic activation of UGT1A1-catalyzed E3G synthesis can be modulated by xenobiotics, such as steroids, flavones and isoflavones [16,25]. Particularly interesting are the effects obtained with 17-alpha-ethynylestradiol, which at low concentrations increases E3G formation preserving the hooked shape of Eadie-Hofstee plot, whereas at higher concentrations markedly inhibits E3G formation shifting the Eadie-Hofstee plot toward a linear relationship typical of Michaelis–Menten kinetics [16].

Thus, the effects on E3G synthesis obtained with repeated *in vivo* exposure to heroin, and to a lesser extent to naltrexone, appear to be similar to those obtained *in vitro* with high concentrations of 17-alpha-ethynylestradiol. The effects of

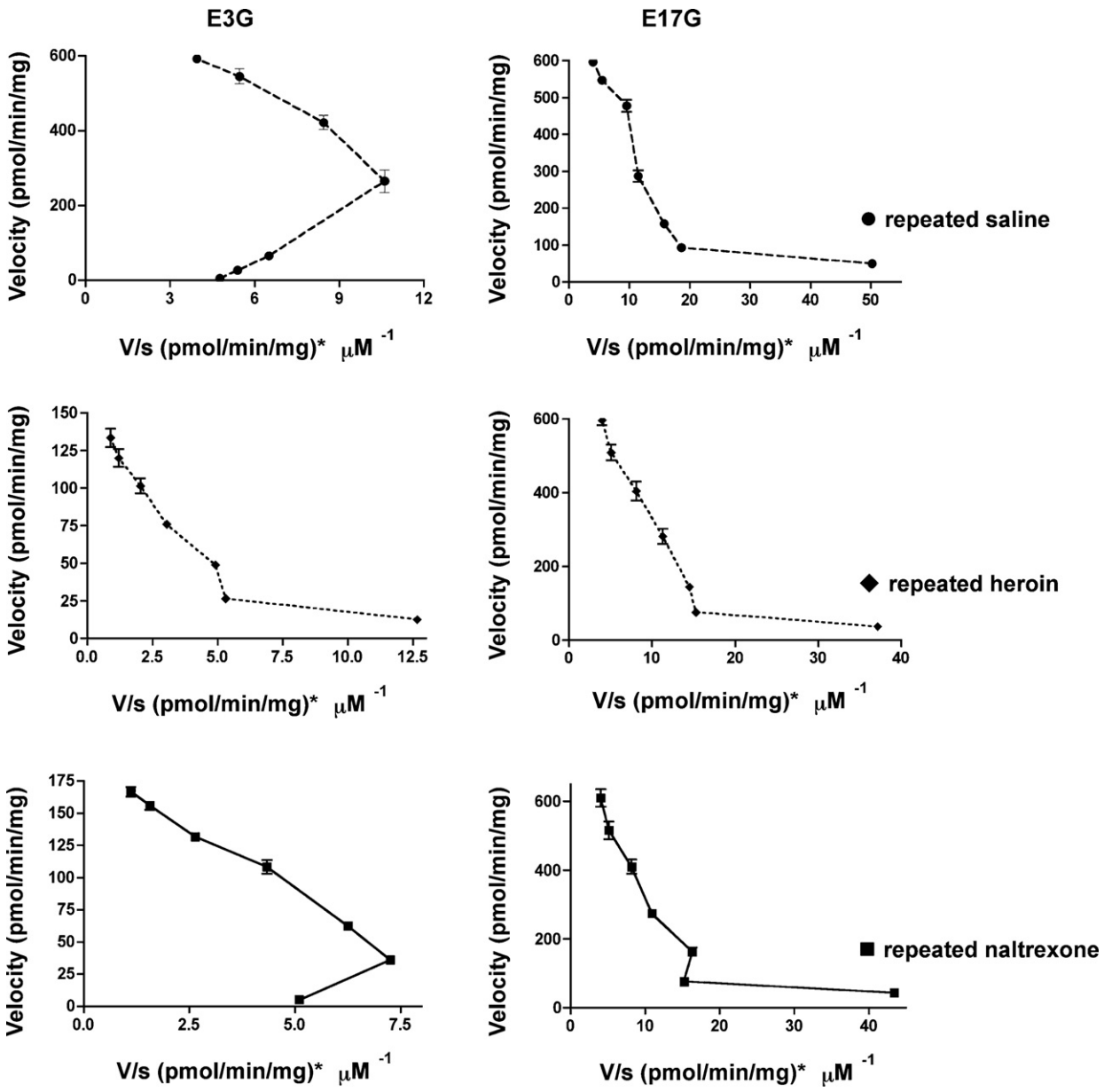

Fig. 2 – Eadie-Hofstee plots of E3G and E17G formation by microsomal preparations from rats treated with saline (upper panels), heroin (middle panels) or naltrexone (lower panels). As expected in control conditions, E3G formation followed the "hooked curve" typical of homotropic activation. Heroin transformed this "hooked curve" in the exponential relationship typical of Michaelis–Menten kinetic, whereas naltrexone shifted the flex point to a higher substrate concentration. These data are replotted from those shown in the graphs of Fig. 1.

this contraceptive agent on the kinetics of E3G formation have been attributed to the occupancy of multimeric units of UGT1A1 [16]. This explanation cannot be invoked in the present study because in our experimental conditions the drugs were absent from the medium when the microsomes were incubated with the substrate.

We have tested here the hypothesis that heroin and naltrexone acted directly on the expression of UGT isoenzymes involved in the formation of estradiol glucuronides. Two lines of evidence support the hypothesis that chronic exposure to heroin or naltrexone may impinge on the expression of UGT genes. First, several drugs have been found to either induce or inhibit UGT expression in the liver through their interaction with specific nuclear receptors [2,26,27]. Second, opioid drugs can influence enzymatic activity by binding nuclear receptors. For instance, nuclear opioid binding sites associated with regulatory protein kinase C have been found by Ventura et al. in cardiac cells [28]. So far, these binding sites have not been detected in the liver, yet there is evidence that other nuclear receptors, such as nuclear pregnane X receptor and constitutive androstane receptors, are involved in the induction of liver UGT activity elicited by phenobarbital- and 3-methylcolanthrene-type inducers [29,30]. However, in the present study heroin and naltrexone

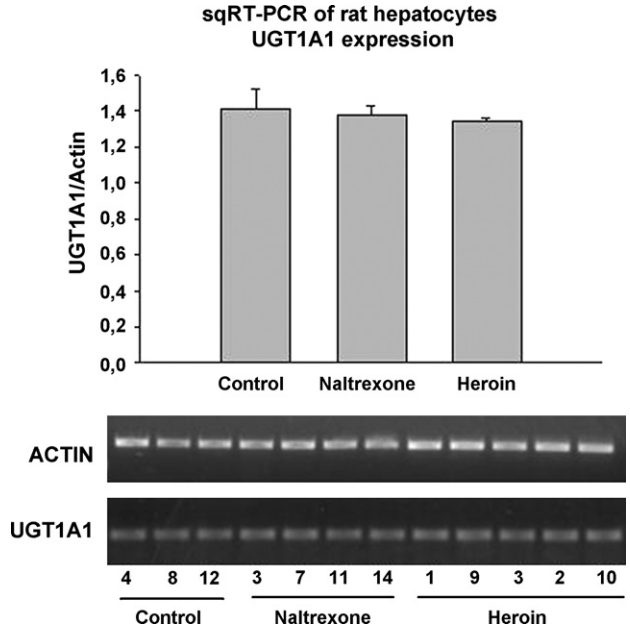

Fig. 3 – mRNA expression of UGT1A1 and Actin in the liver of rats treated daily for 10 days with saline, naltrexone (20 mg/kg, i.p.) or heroin (10 mg/kg, i.p.) (lower panel). In order to assess whether treatment with naltrexone or heroin regulates the expression pattern of UGT1A1, thus correlating with the modification of the kinetics of E3G shown in Fig. 1, semi-quantitative RT-PCR (reverse transcriptase PCR) was performed as described in Section 2. The figure shows no detectable differences between samples treated with drugs and the saline control, suggesting that different mechanisms (as post translational protein control) other than regulation of mRNA expression should be involved in the effects of these drugs on the kinetics of EG3 formation. UGT1A1/Actin mRNA ratio is expressed as mean ± S.E.M. (upper panel).

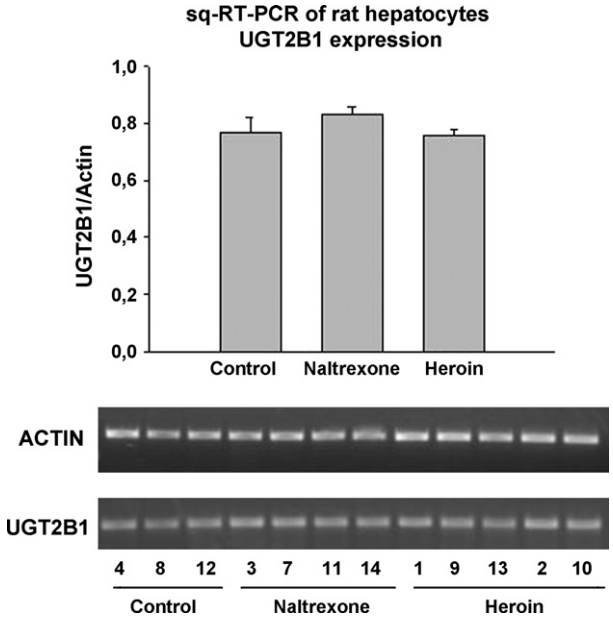

Fig. 4 – mRNA expression of UGT2B1 and Actin in the liver of rats treated daily for 10 days with saline, naltrexone (20 mg/kg, i.p.) or heroin (10 mg/kg, i.p.) (lower panel). In order to assess whether treatment with naltrexone or heroin regulates the expression pattern of UGT2B1, semi-quantitative RT-PCR (reverse transcriptase PCR) was performed as described in Section 2. The figure shows no detectable differences between samples treated with drugs and the saline control, consistent with our previous experiments showing that neither naltrexone nor heroin affected E17G kinetic (Fig. 1). UGT2B1/ACTIN mRNA ratio is expressed as mean ± S.E.M. (upper panel).

had no effect on the expression of the genes encoding for UGT1A1 or UGT2B1.

Although we did not determined the protein expression of UGT1A1 and UGT2B1, our data seem to exclude the possibility that heroin and naltrexone act on the synthesis of these isozymes and suggest that their most plausible mechanism of action on glucuronidation depends on impediment of homotropic UGT1A1 activation. The possible mechanism(s) responsible for this impediment are not clear, but recent findings indicate that post-translational modifications of UGTs are necessary for their catalytic activity. In our experimental situation, these modifications may derive from the interaction of UGT1A1 with other enzymes. In fact, it has been found that the co-expression of human UGT1A4 and UGT1A6 decreases V_{max} of UGT1A1-catalyzed E3G formation [31]. In contrast, the glucuronidation of phenytoin catalyzed by UGT1A1 is enhanced by the co-expression of UGT1A4 [32]. Moreover, adding CYP3A4 to cells expressing UGT2B7 increases the synthesis of the active morphine metabolite M6G at the expenses of M3G formation [33], whereas inhibition of CYP3A4 activity by ketoconazole reduces the UGT2B7-catalyzed formation of both M3G and M6G [34]. Thus, the consequences of the heterodimerization of UGT isozymes depend on the type of substrate involved. This notion is important in order to interpret the overall effects of heroin on glucuronidation. We have already found that either *in vivo* or *in vitro* prolonged exposure to heroin reduces the synthesis of M3G while eliciting the formation of M6G that, under normal conditions, is not synthesized in the rat. It appears that heroin can inhibit, facilitate or leave unchanged the glucuronidation of the hydroxyl function according to its position. In the rat, morphine glucuronidation at the 3-OH position is catalyzed, among the others, by UGT1A1 [35]. A rearrangement of homo- or heterodimerization of UGT1A1 isoenzyme may represent the mechanism by which long-term exposure to phenanthrenic opioids affect morphine as well as estradiol glucuronidation. We will dedicate more studies to elucidate this crucial point.

Finally, it is important to note that MOR does not appear to mediate heroin and naltrexone effects on glucuronidation. We have already observed that heroin modulation of morphine glucuronidation is not shared by the MOR agonist methadone and is not prevented by the MOR antagonist naltrexone [11,12]. Moreover, we have found that naltrexone has intrinsic activity

on the glucuronidation process, increasing the V_{max} of morphine-3-glucuronide synthesis [11] and decreasing the V_{max} of E3G formation. The existence of MOR-independent opioid effects is not surprising. It has been recently reported that morphine can protect rat hepatocytes from anoxia/reoxygenation injury via MOR-independent mechanism [36]. This effect seems to depend on both nitric oxide (NO) synthesis and ATP-sensitive potassium channel activity. Moreover, opioids have been found to modulate antibody and cytokine secretion by multiple myeloma cells in both MOR-dependent and -independent manner [37]. Finally, in the mouse it has been observed that both morphine and its MOR-inactive isomer dextro-morphine can elicit an anti-analgesic effect that is mediated by the direct activation of p38 MAPK [38].

To the best of our knowledge, so far little attention has been paid to the effects of chronic exposure to heroin or to naltrexone on liver glucuronidation in the clinical setting. The fact that intravenous heroin users exhibit reduced blood M3G/M6G ratio relative to opioid-naïve patients treated with morphine encourages us to investigate the mechanisms glucuronidation of hexogen and endogen compounds in these patients. On the basis of results illustrated here, we may indeed predict the presence of altered estradiol glucuronidation in intravenous heroin users, who are characterized by a profound derangement of steroidal hormones turn-over.

Acknowledgments

This study has been supported by grants from the University of Rome ''La Sapienza'' (Finanziamento progetti di ricerca Ateneo) and from the Ministero dell'Istruzione, Università e Ricerca (PRIN-2006050704).

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