Bioactivation of Morphine in Human Liver: Isolation and Identification of Morphinone, a Toxic Metabolite

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Morphinone, identified in the bile of guinea pigs and rats given morphine, is a reactive electrophile and has the ability to bind to glutathione (GSH) and tissue macromolecules, leading to GSH depletion and cell damage. We previously demonstrated that the livers of various animal species are capable of forming morphinone from morphine. In this study, we examined whether the human liver can produce morphinone from morphine. HPLC analysis revealed that the incubation of morphine with the 9000 × g supernatant of human liver in the presence of NAD(P) and 2-mercaptoethanol (ME) gave a peak corresponding to the synthetic morphinone-ME adduct (MO-ME), which is readily formed by a nonenzymatic reaction of morphinone with ME. The reaction product was isolated and was unambiguously identified as MO-ME using FAB-MS and NMR analyses in comparison with synthetic MO-ME. The conversion of morphine to morphinone required NAD(P), and NAD was a preferred cofactor over NADP. All the 9000 × g supernatants from six human livers could produce morphinone at different rates, ranging from 30 to 120 nmol/g liver/30 min with NAD at pH 7.4. The enzyme activity responsible for the formation of morphinone from morphine was mainly localized in the microsomes. The microsomal enzyme activity was inhibited by steroids, lithocholic acid and indomethacin. Among these compounds, steroids with a 17β-hydroxyl group almost completely depressed morphinone formation. In conclusion, the metabolic pathway of morphine to morphinone, a toxic metabolite, in human liver was shown for the first time in in vitro experiments.

Key words morphine metabolism; morphinone; metabolic activation; morphine 6-dehydrogenase; human liver

Morphine is a potent analgesic and is used worldwide in the clinical management of severe acute and chronic pain. It is known, however, that the repeated administration of morphine results in tolerance and physical dependence. It has been shown that the treatment of the rat and mouse with high doses of morphine decreases the hepatic glutathione (GSH) content and increases the levels of serum transaminase activity.1–3) These adverse reactions of morphine have long been thought to be due, at least in part, to the generation of reactive metabolite(s) that can bind to GSH and tissue macromolecules. We found morphine 6-dehydrogenase, which catalyzes the conversion of morphine to morphinone in the guinea pig livers and purified the enzyme.4) The product with the enzyme was identified as the 2-mercaptoethanol (ME) adduct of morphinone (MO-ME) which was formed by nonenzymatic binding of morphinone to ME in the incubation medium via a Michael addition, indicating that morphinone is a reactive electrophile. This finding led us to the identification of the morphinone-GSH adduct (MO-GSH) in the bile of guinea pigs given morphine.5) Thus the metabolic pathway of morphine to morphinone and subsequently to the MO-GSH was first demonstrated in the guinea pig (Fig. 1).

The latter reaction was considered to occur mainly through a nonenzymatic binding of morphinone to GSH and, in part, in a reaction catalyzed by the GSH S-transferase.6) In vivo studies in the guinea pig6) and rat7) showed that most morphinone formed from morphine is commonly excreted as MO-GSH into the bile and that the sum of morphinone and MO-GSH accounted for about 10% in the guinea pig and 8% in the rat of the administered dose. These observations indicate that the pathway of morphine to morphinone catalyzed by morphine 6-dehydrogenase is one of the main routes in morphine metabolism, at least in guinea pigs and rats.

In the mouse, morphinone is nine-fold more toxic than morphine2) and is a potent antagonist of morphine.8) This metabolite was also found to block naloxone binding irreversibly in the mitochondrial-synaptosomal fractions of the mouse brain;9) to bind covalently to tissue macromolecules through sulfhydryl groups in the mouse,9) and to cause hepatotoxicity in the rat.10)11) These observations suggest that morphinone formed from morphine may affect the analgesic action of morphine and has a potential role in morphine-induced toxicity including the development of tolerance, although most morphinone would be trapped by intracellular GSH to form MO-GSH and excreted readily into the bile. It is therefore of toxicologic and pharmacologic interest to assess the metabolic pathway of morphine to morphinone in animals and humans.

We previously reported that the livers of rabbit, mouse, hamster, and bovine as well as guinea pig and rat have the ability to produce morphinone from morphine.1) However, the in vivo and in vitro formation of morphine and MO-GSH,
GSH in humans has not been reported. Therefore the present study was undertaken to elucidate the metabolic pathways of morphine to morphinone in humans in an in vitro experiment. We provide evidence that the human liver is capable of forming morphinone from morphine. We also describe some features of morphine 6-dehydrogenase activity in the human liver and compare it with that in other mammalian livers reported previously.

MATERIALS AND METHODS

Materials Morphine hydrochloride was obtained from Takeda Chemical Ind., Ltd. (Osaka, Japan), pyridine nucleotides from Oriental Yeast Co., Ltd. (Osaka), and steroids, naloxyphosphate, and indomethacin from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Morphinone was synthesized by the method of Rapoport et al.12) MO-ME was synthesized as described previously.4) All other chemicals used were of the highest grade available.

Human Liver and Enzyme Preparation Human livers were obtained from Laboratory of Forensic Medicine, Fukuoka University School of Medicine after postmortem examinations (within 20 h after death) and stored at −80°C until use. Livers were homogenized in two volumes of sodium phosphate buffer 0.1 M, pH 7.4, with a Piriton homogenizer (Kinematika GmbH, Luzen, Switzerland). To yield the 9000 × g supernatant, microsome, and cytosol fractions, the homogenate was successively centrifuged at 9000 × g for 20 min and at 105000 × g for 60 min, and the resulting pellet (microsomes) was washed twice with 0.33 g wet liver weight/ml with sodium phosphate buffer 0.1 M, pH 7.4.

Isolation and Identification of Morphinone as MO-ME The reaction mixture consisted of morphine (3 mM), NAD or NADP (6 mM), the enzyme fraction (equivalent to 0.165 g of wet liver), ME (3 mM), and sodium phosphate buffer 0.1 M, pH 7.4, in a total volume of 3 ml and was incubated at 37°C for 30 min. ME was added to trap morphinone as the stable MO-ME. The reaction was initiated by the addition of the enzyme fraction and was terminated by the addition of 0.3 ml of 20% trichloroacetic acid. Then the reaction mixture was neutralized by adding 2 ml of saturated sodium pyrophosphate and centrifuged at 2000 rpm for 20 min. The resulting supernatant was poured onto a Sep-Pak C18 cartridge (Waters Division of Millipore Co., Milford, MA, U.S.A.). The cartridge was washed with 10 ml of water and eluted with 2 ml of methanol. The eluate was used for quantification of morphinone by reverse-phase HPLC on a column of YMC-Pack ODS-ALL (150×6 mm, YMC Co., Kyoto, Japan) using a mobile phase consisting of sodium phosphate buffer 10 mM, pH 7.2/acetonitrile/methanol (50:45:5, v/v/v) at a flow rate of 1.2 ml/min. The HPLC apparatus consisted of a L-6200 Intelligent Pump, a L-4200 UV-VIS Detector, and a L-6200 Intelligent Pump, a L-4200 UV-VIS Detector, and an L-6200 Intelligent Pump, a L-4200 UV-VIS Detector. The peaks were monitored for absorbance at 214 nm and the peak area was determined on a D-2500 Chromato-Integrator (Hitachi Ltd.). Under these conditions, the retention time of MO-ME was 6.5 min. The mean recovery of morphinone was 93% (n = 3).

Isolation of Reaction Product To isolate the reaction product, a large-scale incubation (120 ml) was performed for 2 h at 37°C. The reaction mixture consisted of the 9000 × g supernatant fraction (equivalent to 6.7 g of human liver), 0.1 mM glycine–NaOH buffer, pH 10, morphine (3 mM), NAD (6 mM) and ME (3 mM). After incubation, the reaction mixture was treated as described above. The supernatant obtained after centrifugation was divided into small portions, and each portion was separately treated with a Sep-Pak C18 cartridge as described above. The combined eluate was evaporated under N2 gas at reduced pressure. The resulting residue was poured onto a preparative reverse-phase column (350×24 mm) packed with Deversol ODS 24S (Nomura Chemical Co., Aichi, Japan) and eluted with sodium phosphate buffer 10 mM, pH 3.8, containing tetramethylammonium 1 mM/acetonitrile (9:1, v/v) at a flow rate of 3 ml/min. The fractions containing the reaction product, which was detected by HPLC, were combined and concentrated under N2 gas at reduced pressure. The resulting residue was further treated with a Sep-Pak C18 cartridge. The eluate was evaporated under N2 gas at reduced pressure and then treated with absolute ethanol to yield an amorphous powder.

FAB-MS and NMR Analyses FAB-MS spectra were obtained with a model JMS-HX 110 (JEOL Ltd., Tokyo, Japan). 1H-NMR spectra were obtained with a model FX-400 (JEOL Ltd.) using TMS as an internal standard at 400 MHz.

RESULTS

Isolation and Identification of Morphinone as MO-ME We first examined whether the human liver has the ability to produce morphinone from morphine using the 9000 × g supernatant (prepared from HL1 in Table 1) using HPLC procedures. The incubation at pH 7.4 in the presence of NAD and ME gave a peak at the retention time of 6.5 min (Fig. 2A), which corresponded to synthetic MO-ME. This peak was not observed without morphine in the reaction mixture (Fig. 2B), and a negligible peak was detected in the absence of NAD (Fig. 2C). Using NADP instead of NAD, similar results were obtained but the peak at 6.5 min was about one-third that with NAD (see Table 1). Incubation with the boiled 9000 × g supernatant also gave a negligible peak, suggesting that a trace amount of morphinone formed in the absence of NAD(P) was generated by a hydroxyl radical-mediated reaction, as reported by Kumagai et al.13) When the incubation without ME was carried out and ME was added after incubation, the peak area at the retention time of 6.5 min was rel

<table>
<thead>
<tr>
<th>Human liver</th>
<th>Sex</th>
<th>Age</th>
<th>Morphinone formed (nmol/g liver/30 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL1</td>
<td>F</td>
<td>36</td>
<td>117.6 ± 8.1</td>
</tr>
<tr>
<td>HL2</td>
<td>F</td>
<td>48</td>
<td>128.6 ± 6.0</td>
</tr>
<tr>
<td>HL3</td>
<td>M</td>
<td>23</td>
<td>108.8 ± 8.6</td>
</tr>
<tr>
<td>HL4</td>
<td>M</td>
<td>46</td>
<td>114.3 ± 5.8</td>
</tr>
<tr>
<td>HL5</td>
<td>M</td>
<td>36</td>
<td>30.7 ± 4.5</td>
</tr>
<tr>
<td>HL6</td>
<td>M</td>
<td>57</td>
<td>56.2 ± 6.2</td>
</tr>
</tbody>
</table>

Incubations and quantification of morphinone formed were carried out as described in Materials and Methods. Each value represents the mean ± S.D. of triplicate determinations.
Reduced to about 70%. This reduction is considered to be due to decomposition and/or binding of the morphinone formed to proteins during the incubation.

To verify morphinone formation unequivocally, we next isolated the product and elucidated its structure using FAB-MS and NMR spectrometric analyses. In this experiment, incubation was performed at pH 10, at which the amount of product was about five-fold that at pH 7.4. Using the isolation procedure described in Materials and Methods, sufficient quantities of product for structural analyses were obtained.

As shown in Fig. 3A, the FAB-MS spectrum of the product gave a molecular ion peak at M/Z 362 based on a protonated molecular ion (MH$^+$), identifying the molecular mass as 361. This molecular ion gave a daughter ion at M/Z 284, which represents (MH$^+$/H). These data indicate that the product is the MO-ME adduct. In addition, the spectrum of the product was identical to that of synthetic MO-ME (Fig. 3B). The $^1$H-NMR spectra of the product and of synthetic MO-ME are shown in Fig. 4. The two spectra were essentially identical. The signal at 4.9 ppm, which appeared as a singlet, corresponds to a C-5 proton, indicating that CH–OH at C-6 is replaced by -O. The multiplet signals at 2.63, 2.57, and 2.44 ppm, which overlapped with other multiplet signals, should correspond to C-7α, C-7β, and C-8 protons, respectively. The signals corresponding to protons of the ME moiety were observed at 2.70 and 3.69 ppm. These assignments suggest that the product has a ketone group at C-6 and a saturated bond at C-7 and C-8, and the site of attachment to ME is C-8. From these spectral analyses, the isolated product was unambiguously identified as 8-(2-hydroxyethylthio)dihydromorphinone (MO-ME).
Quantification of Morphinone Formed from Morphine in Human Liver  As described above, the 9000×g supernatant prepared from human liver sample 1 (HL1) produced morphinone from morphine in the presence of either NAD or NADP. Morphinone formation by the other five human livers (HL2—HL6) was also investigated using the 9000×g supernatant using HPLC procedures. All the human livers were capable of forming morphinone from morphine in the presence of either NAD or NADP at a physiologic pH of 7.4 (Table 1). The amount of morphinone formed with NAD was greater than that with NADP in all livers. However, an interindividual difference in the morphinone formation was observed with either cofactor.

The 9000×g supernatants of HL1, HL2, and HL3 were divided into the cytosolic and microsomal fractions, and subcellular distribution of the enzyme activity responsible for the formation of morphinone from morphine was examined. As shown in Fig. 5, the subcellular distribution of the enzyme activity was similar in the three livers. The enzyme activity with NAD was mainly localized in the microsomal fraction, whereas that with NADP was slightly higher in the cytosolic fraction than in the microsomal fraction. The rates with NADP in both fractions were about one-fifth of those with NAD in the microsomal fraction. Thus morphinone formation in human liver was catalyzed mainly by the enzyme(s) present in the microsomal fraction, with a preference for NAD. It is noted that enzyme activity with NAD in the microsomal fraction was slightly higher (about 1.2-fold) than that observed in the 9000×g supernatant in the three livers.

Characterization of Microsomal Morphine 6-Dehydrogenase Activity  We also characterized the enzyme activity using the microsomal fraction of HL1 with respect to pH dependency, kinetic parameters, and susceptibility to inhibitors.

When enzyme activity was determined at various pH values (pH 6.5 to 8, sodium phosphate buffer; pH 7 to 9, Tris–HCl buffer; and pH 8.5 to 10.5, glycine–NaOH buffer), the maximum activity was observed at around pH 9.8. To assess the kinetic parameters under linear conditions for morphinone formation, 30-min incubation time was chosen and enzyme activity with various concentrations of morphine (0.5—8 mM) was determined. The $K_m$ and $V_{max}$ values for morphinone formation estimated using Lineweaver–Burk plots were 5.5±0.8 mM and 231±15 nmol/g liver/30 min ($n$ = 3), respectively.

Finally, we examined the inhibitory effects of various compounds on the enzyme activity. The results obtained are summarized in Table 2. Indomethacin (1 mM) and various steroids (0.1 mM) potently inhibited morphinone formation. Lithocholic acid (0.1 mM), a monohydroxyl bile acid, also acted as a potent inhibitor, but the inhibitory potency of bile acid decreased as the number of hydroxyl groups in the molecule increased. Naloxone (1 mM), barbital (1 mM), and ketamine (1 mM) inhibited morphinone formation moderately. Interestingly, steroids with a 17β-hydroxyl group were the most powerful inhibitors and almost completely suppressed morphinone formation at a concentration of 0.1 mM.

DISCUSSION

The metabolism of morphine has been extensively studied in various animal species and humans, and consequently a number of metabolites have been identified in vivo and in vitro experiments.[14] Although the pattern of morphine metabolism is species dependent, the main route is commonly glucuronidation at C-3 to form morphine 3-glucuronide. In

![Fig. 5. Subcellular Distribution of Morphine 6-Dehydrogenase Activity in Human Liver](image)

**Fig. 5.** Subcellular Distribution of Morphine 6-Dehydrogenase Activity in Human Liver

Incubations and quantification of morphinone formed were carried out as described in Materials and Methods. Data represent the mean±S.D. of triplicate determinations.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (mM)</th>
<th>Morphinone formed (nmol/g liver/30 min)</th>
<th>Inhibition rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrazole</td>
<td>1.0</td>
<td>117.6±5.9</td>
<td>20</td>
</tr>
<tr>
<td>Barbitol</td>
<td>1.0</td>
<td>47.0±10.0</td>
<td>68</td>
</tr>
<tr>
<td>Naloxone</td>
<td>1.0</td>
<td>57.5±6.4</td>
<td>67</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>1.0</td>
<td>23.9±1.3</td>
<td>84</td>
</tr>
<tr>
<td>Ketamine</td>
<td>1.0</td>
<td>58.9±7.7</td>
<td>60</td>
</tr>
<tr>
<td>Testosterone</td>
<td>0.1</td>
<td>—[a]</td>
<td>100</td>
</tr>
<tr>
<td>5α-Dihydrotestosterone</td>
<td>0.1</td>
<td>—[a]</td>
<td>100</td>
</tr>
<tr>
<td>5β-Dihydrotestosterone</td>
<td>0.1</td>
<td>—[a]</td>
<td>100</td>
</tr>
<tr>
<td>Androsterone</td>
<td>0.1</td>
<td>34.8±2.3</td>
<td>76</td>
</tr>
<tr>
<td>Epiandrosterone</td>
<td>0.1</td>
<td>33.4±3.9</td>
<td>77</td>
</tr>
<tr>
<td>Estradiol</td>
<td>0.1</td>
<td>—[a]</td>
<td>100</td>
</tr>
<tr>
<td>Lithocholic acid</td>
<td>0.1</td>
<td>36.2±3.7</td>
<td>75</td>
</tr>
<tr>
<td>Chenodeoxycholic acid</td>
<td>0.1</td>
<td>111.6±12.1</td>
<td>24</td>
</tr>
<tr>
<td>Cholic acid</td>
<td>0.1</td>
<td>132.7±13.2</td>
<td>10</td>
</tr>
</tbody>
</table>

[a] Negligible peak almost equal to that obtained without NAD in the reaction mixture.

Morphine (3 mM) was incubated with human liver microsomes from HL1 (equivalent to 0.165 g of liver) in the presence of either NADP or NAD as cofactor for 30 min at pH 7.4. Extraction and quantification of morphinone were carried out as described in Materials and Methods. Each value represents the mean±S.D. of triplicate determinations. The amount of morphinone formed in the absence of inhibitor was 147.7±9.6 nmol/g liver/30 min ($n$ = 3).
humans, glucuronidation at C-6, N-demethylation, and sulfate conjugation at C-3 have also been reported as minor routes. In the present study, after incubation of morphine with human liver in the presence of NAD and ME, we isolated the reaction product and identified it as MO-ME, which was generated by trapping morphinone formed from morphine with ME, using FAB-MS and NMR analyses. In addition, all the livers used in this study were able to produce morphinone in the presence of either NAD or NADP at a physiologic pH of 7.4. Thus the presence of a metabolic pathway of morphine to morphinone in humans has been unequivocally demonstrated in vitro experiments. To our knowledge, the present study is the first case in which morphine was biotransformed into a reactive metabolite that has the potential to deplete GSH and to bind covalently to tissue proteins in humans.

Bioactivation of drugs to the reactive metabolites is thought to be the primary step in many forms of chemical toxicity, although the precise mechanism underlying drug-induced toxicity has not yet been clarified. Nagamatsu et al., and Nagamatsu and Hasegawa provided convincing evidence for the involvement of the hepatic metabolism of morphine to morphinone in morphine-induced hepatotoxicity using isolated rat hepatocytes. The formation of MO-GSH from the intermediate metabolite morphinone occurred along with a depleted cellular concentration of GSH and decreased viability of hepatocytes. They detected morphinone-protein adducts, particularly when the hepatocytes were damaged. They also showed that the addition of naloxone, an inhibitor of the rat microsomal morphine 6-dehydrogenase, decreased the formation of MO-GSH and prevented morphine-induced hepatocyte toxicity. Accordingly, the present study demonstrating the hepatic metabolism of morphine to morphinone in humans is of clinical significance and importance as morphine is widely used for analgesic purposes.

Recently, Zhang et al. have suggested the involvement of oxidative stress in morphine-induced hepatotoxicity in mice. The administration of morphine increased the oxidative stress indexes such as that of 8-hydroxydeoxyguanosine, the protein carbonyl group, and malondialdehyde, and decreased the ratio of GSH to GSSG in the liver. They also showed that the injection of antioxidants such as GSH and ascorbic acid attenuated these responses and prevented morphine-induced hepatotoxicity. In this connection, we previously showed that the administration of morphine to mice depletes GSH in the liver. This finding suggests that the metabolism of morphine to morphinone may also participate, at least in part, in the induction of oxidative stress by depleting GSH through the formation of MO-GSH in the liver.

We previously showed that the guinea pig, rat, rabbit, mouse, and bovine livers contain the enzyme activity responsible for the formation of morphinone from morphine and that NAD was a more efficient cofactor than NADP except in guinea pig liver, which equally utilized both cofactors. However, subcellular distribution of the enzyme activity was different among animal species. The enzyme activity in the rat was mainly distributed in the microsomal fraction, whereas the guinea pig, hamster, and bovine expressed the enzyme activity mainly in the cytosolic fraction. The rabbit and mouse showed considerable activity in both fractions. The present study demonstrated that the formation of morphinone from morphine in human liver was mediated mainly by the microsomal enzyme(s) with a preference for NAD over NADP. This feature is similar to that in the rat liver. In this study, we characterized some properties of the enzyme activity in the human liver microsomes to compare them with those in the rat liver microsomes described previously. The $K_m$ values for morphine and pH optima of morphinone formation in the human and rat liver microsomes did not differ markedly; the $K_m$ values were 5.5 mM in human and 3.3 mM in rat microsomes, and pH optima were 9.8 in human and 9.3 in rat microsomes. Inhibition experiments revealed that most of the compounds tested had similar inhibitory effects on the human and rat microsomal enzyme activities, and that hydroxysteroids and lathicholic acid act as powerful inhibitors. These findings, together with the fact that the cytosolic enzymes in the guinea pig, rabbit, and hamster livers belong to the aldo-keto reductase superfamily, suggest that the microsomal enzymes in human and rat livers are structurally related proteins.

We are currently purifying the microsomal enzyme from rat liver. Although the enzyme has not yet been obtained as a homogeneous protein, morphine 6-dehydrogenase activity and 17β-hydroxysteroid dehydrogenase (17β-HSD) activity using testosterone as substrate coeluted with each other at all stages in the purification procedure, suggesting that the rat enzyme is identical to 17β-HSD. In this connection, Akinola et al. have cloned a cDNA for microsomal 17β-HSD, referred to as rat 17β-HSD type 2, with a preference for NAD as a cofactor in the oxidation of testosterone from a rat placental library. They also showed that rat 17β-HSD type 2 is expressed in the liver as well as placenta. The corresponding human enzyme (human 17β-HSD type 2) that has 62% amino acid sequence identity to rat 17β-HSD type 2 has been identified in the human liver. Accordingly, it is proposed that the human 17β-HSD type 2 as well as the rat isoform may participate in the formation of morphinone from morphine. Strong inhibition by 17β-hydroxysteroids observed in this study may be the result of their competition with morphine at the substrate-binding site of the enzyme (human 17β-HSD type 2).

In conclusion, the present study unequivocally demonstrated that the human liver can produce morphinone from morphine. Considering the slow rate and high $K_m$ value for morphinone formation in the human liver, it is likely that the metabolic pathway of morphine to morphinone may be a minor route when used in a small dose, and subsequently the low concentrations of morphinone formed are readily detoxified by GSH. However, morphine is often administered in high doses for analgesic purposes and concurrently with other drugs that are metabolized by the UDP-glucuronyl transferase or may depend on GSH for detoxification. Under these circumstances, the glucuronide formation pathway, a main route in morphine metabolism, may become saturated or GSH in the liver may be depleted, and consequently a significant elevation in the level of morphinone may occur, resulting in morphine toxicity.

REFERENCES