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Comparative evaluation of different pathways for the liver toxicity of morphine using freshly isolated hepatocytes

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Hepatic injury has been observed in patients that are treated with narcotics. Thureson-Klein *et al.* [1] indicated that implantation of morphine pellets in mice caused hepatic fatty infiltration and increases in serum transaminases. Chang and Ho [2] and Needham *et al.* [3] showed that intraventricular injection of morphine caused an increase in serum GOT and GPT in mice and suggested that the hepatic injury by morphine was mediated via CNS mediated pathways.

Recently, Nagamatsu *et al.* [4] indicated that morphine 6-dehydrogenase in cytosolic fraction of mouse liver metabolised morphine to chemically unstable morphinone which reacted with a cellular reduced form of glutathione (GSH) and macromolecules. On the other hand, Correia *et al.* [5] showed microsomal cyt. P-450 mediated metabolic activation of morphine.

On comparing these two pathways for morphine toxicity using freshly isolated rat hepatocytes we showed the relative importance of the reaction mediated by morphine 6-dehydrogenase.

Materials and methods

Isolated hepatocytes were prepared from male rats (Wistar strain, 9–13 weeks, Nihon rat Co.) as described by Moldeus *et al.* [6] and used immediately. The viability of the hepatocytes, determined by trypan blue exclusion, was 92–97%. Hepatocytes were suspended in Krebs–Henseleit buffer supplemented with 12.5 mM HEPES (10^6 cells/ml), and these cells were incubating in rotating round-bottom siliconized glass flasks at 37° under continuous gassing with carbogen (95% O_2, 5% CO_2). Changes in viability during incubation were determined by measuring the leakage of lactate dehydrogenase from the cells [7] (initial values were 77–92%).

Cellular glutathione (GSH) was determined spectrofluorometrically [8] after centrifugation of the cell suspension at 8000 rpm for 5 sec. GSH contents were confirmed by using high performance liquid chromatography (HPLC). This method was originally described by Toyo'oka and Imai [9]. In the case of determination of morphine metabolism, the cell suspensions were frozen and thawed. After centrifugation, the supernatants were analyzed by HPLC [10].

Covalent binding of morphine to the macromolecules were determined after the incubation of hepatocytes with ^{3}H-morphine (0.5 mM, 0.15 μCi/mole) at 37°.

^{3}H-morphine (50.6 Ci/mmole) was purchased from NEN Research Products (Boston, MA). All other chemicals used were of at least reagent grade and obtained from local commercial sources.

Results and discussion

As described before [10], cellular GSH and viabilities were decreased by the addition of morphine (0.5 mM) to the hepatocyte suspension (Table 1).

Metyrapone, an inhibitor of cyt. P-450 linked drug metabolism, inhibited the GSH decrease by morphine a little (Table 1). In some cases, it slowed the rate of GSH depletion by about 30% at 30 min of incubation and inhibited the loss of cell viability by morphine. On the other hand, piperonyl butoxide, which also inhibits cyt. P-450 linked drug metabolism, slowed the GSH depletion a little, but potentiated the effects of morphine on the cell viability. Metyrapone and piperonyl butoxide themselves had no effects on the hepatocytes. SKF 525-A and α-naphthoflavone did not inhibit the toxicity at 0.1 mM. These drugs were toxic to the hepatocytes when the concentrations were higher than 0.25 mM. Morphine also decreased the GSH content of hepatocytes rapidly in which cyt. P-450 was decreased by the pretreatment of rats with $CoCl_2$ (40 mg/kg) (results were not indicated). From these results, we can interpret that the participation of the cyt. P-450 mediated pathway plays a minor role in the hepatocyte toxicity of morphine.

Table 1. Effects of various inhibitors on the decrease of cellular GSH and viability by morphine (0.5 mM)

	GSH content at 30 min (nmole/10^6 cells)	Viability at 3 hr (%)
Control	42.5 ± 1.4 (19)	62.4 ± 2.3 (18)
Morphine	8.1 ± 1.2 (19)	6.7 ± 3.3 (16)
Morphine + metyrapone	11.7 ± 2.7 (9)	13.7 ± 3.0 (8)
Morphine + naloxone	15.1 ± 4.2 (5)	43.8 ± 3.8 (5)
Morphine + naltrexone	34.1 ± 1.3 (4)	55.4 ± 7.1 (4)

These values indicate mean ± SEM and numerals in parentheses indicate the number of batch of hepatocytes.

Concentration of morphine was 0.5 mM and that of inhibitors was 1 mM.

The effects of morphine antagonists, naloxone and naltrexone, were also examined. Naloxone was reported to inhibit morphine 6-dehydrogenase [11]. However, inhibition of morphine-induced GSH decrease by naloxone was weak. On the other hand, naltrexone inhibited it by about 75%. These drugs inhibited the loss of cell viability. The effects were more pronounced also in naltrexone than in naloxone. The difference between naloxone and naltrexone seemed to be based on the fact that naltrexone was metabolically more stable than naloxone.

Inhibitors of alcohol dehydrogenase (E.1.1.1.1: pyrazole, tetramethylene glutarate, phenobarbital [12, 13]) and aldehyde oxidase (E.1.2.3.1: phenobarbital [13]) had no effects on the toxicity (results were not indicated).

Macromolecular binding of morphine was also observed when morphine was incubated in a suspension of freshly isolated hepatocytes. Even though cellular GSH was not depleted at 30 min after incubation, the binding was observed. These binding were inhibited by naloxone and naltrexone (results were not indicated) in almost similar manner. The inhibition by metyrapone was not pronounced.

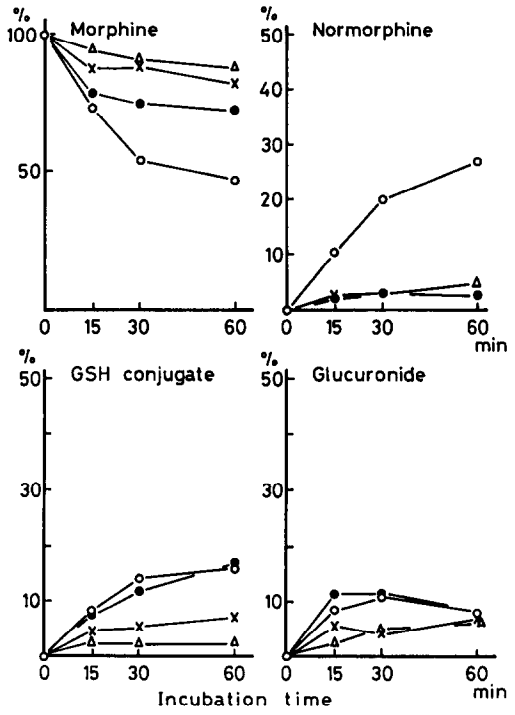

Fig. 1. Effects of metyrapone and naltrexone on the metabolic conversion of morphine: ○, control cell; ●, metyrapone (0.5 mM); ×, naltrexone (0.5 mM); △, metyrapone (0.5 mM) plus naltrexone (0.5 mM). Initial concentration of morphine was 0.25 mM and the results were indicated as per cent of total morphine equivalent (ordinate).

Incubation of hepatocytes with morphine resulted in the formation of normorphine, morphine glucuronide and morphine glutathione (Fig. 1). Metyrapone inhibited the formation of normorphine and GSH conjugate by about 86% and 20% at 30 min of incubation, respectively. Naltrexone inhibited the formation of normorphine, GSH conjugate, and glucuronide by about 85%, 65% and 64%, respectively. When metyrapone and naltrexone were used together, they were inhibited by about 85%, 86% and 55%, respectively. Formation of normorphine was also inhibited by both metyrapone and naltrexone. However, normorphine did not have any toxicity in isolated hepatocytes [10]. Because GSH conjugates can be formed by nonenzymatic reaction of GSH with morphinon, which is formed from morphine by morphine 6-dehydrogenase [10, 11], these results seemed to indicate that naltrexone suppressed the toxicity of morphine by the inhibition of the enzyme.

In conclusion, results obtained by using freshly isolated hepatocytes suggest that microsomal cyt. P-450 linked drug metabolism plays, at the most, a minor role in the hepatocyte toxicity of morphine in rats and that the other enzyme, probably morphine 6-dehydrogenase, plays a major role in the toxicity.

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