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HEPATOTOXICITY OF THE OPIOIDS MORPHINE, HEROIN, MEPERIDINE, AND METHADONE TO CULTURED HUMAN HEPATOCYTES

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Adult human hepatocytes in chemically defined culture conditions were incubated with morphine, heroin, meperidine, and methadone to investigate their potential hepatotoxicity to human liver. Cytotoxic effects were observed at about 100 times the plasma concentrations required to produce analgesia in human nonaddicts. Concentrations of 1 mM morphine, heroin, and meperidine reduced the glycogen content by 50%, while even 0.2 mM methadone produced a depletion of 70% after 24 h of treatment. Concentrations of 0.8 mM morphine and heroin, 0.4 mM meperidine, and 0.005 mM methadone inhibited the albumin synthesis by about 50% after 24 h of pretreatment. Intracellular glutathione was reduced to 50% of that of controls after 2–3 h of incubation with 2 mM morphine and 1 mM heroin, while 1 mM meperidine and 0.2 mM methadone produced a reduction of about 30% after 6 h incubation. The results show that therapeutic dosis of the opioids is unlikely to produce irreversible damage to human hepatocytes, but opiate dosis during tolerance or abuse may be a cause of liver dysfunction.

INTRODUCTION

Opioids are a group of natural and synthetic drugs sharing properties with central nervous system enkephalins and endorphins, and exhibiting varying degrees of morphinelike pharmacological effects. Morphine, meperidine, and methadone are of clinical interest. Their therapeutical use is, however, restricted to situations where a strong analgesia is needed or

The authors wish to express their gratitude to Fondo de Investigaciones Sanitarias de la S.S., and to the Diputación General de Valencia for financial support. X. Ponsoda and R. Jover were recipients of fellowships from FISS and M.E.C., respectively.

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Molecular Toxicology, 1:453–463, 1987

as a complement to anesthesia. Methadone, a long-acting opioid, is actually of generalized use to counteract the heroin withdrawal syndrome. Heroin is the most frequently abused drug, and methadone, although aimed to counteract addiction to heroin, is also frequently abused.

Liver pathology among opiate addicts is very common. Its pathogenesis has been debated for many years. It was formerly believed that liver alterations in drug addicts were the result of previous viral infection, impurities of the self-administered drug, or alcohol abuse (Gorodetzky et al., 1968). However, experimental evidence has shown that opiates deplete hepatic glutathione (James et al., 1982; Eklöw-Låstbom et al., 1986), induce hyperglycemia (Feldberg and Shaligram, 1972; Ipp et al., 1980), and elevate serum transaminases in animals (Chang and Ho, 1979). It has also been shown that morphine is toxic to rat hepatocytes. Clinical studies have documented liver dysfunction attributed to morphine and heroin (McEvoy, 1987) as well as methadone (Kreek et al., 1972). These results indicate that opioids may be intrinsic hepatotoxins and point to the potential risk of hepatotoxicity for humans after chronic therapy or self administration. Of particular interest is the previous observation showing methadone to be toxic to mice (Hui et al., 1978) and rat hepatomas in vitro (Beverley et al., 1984).

In order to investigate the relevance of these observations to human liver, we exposed human hepatocytes obtained from liver biopsies, cultured in chemically defined conditions, to morphine, heroin, meperidine, and methadone, and investigated their cytotoxic as well as metabolic effects on these cells. Our results show that among the opiates being investigated, methadone is the most hepatotoxic compound, exerting its toxic effects in vitro at concentrations that are likely to be reached in vivo during drug misuse and therapy.

MATERIALS AND METHODS

Isolation and Culture of Human Hepatocytes

Surgical liver biopsies (1–3 g) were obtained from patients undergoing cholecystectomy after informed consent was obtained. Hepatocytes were isolated by a two-step perfusion technique. During the first step, the tissue was perfused with a calcium-free buffer (1.37 m*M* NaCl, 2.68 m*M* KCl, 0.7 m*M* $N_2PO_4H \cdot 12H_2O$, 10 m*M* HEPES, 10 m*M* glucose, and 0.5 m*M* EGTA, pH 7.5) at a flow rate of 10 ml/min·catheter without recirculation. The second step was a recirculating perfusion with the same buffer without EGTA but with 5 m*M* $CaCl_2$ and 0.5 mg/ml collagenase (Boehringer, specific activity 0.35 U/mg), at the same flow rate for about 30 min. The perfusion buffers were continuously oxygenated. The cellular suspension was washed twice in ice-cold buffer and centrifuged for 2 min at 50 × g. The cellular viability was esti-

mated by the dye exclusion test with 0.4% trypan blue in saline. Cells were resuspended in Ham F-12 medium supplemented with 2% newborn calf serum, 50 U/ml penicillin, 50 μg/ml streptomycin, 0.2% BSA, and 10^{-8} *M* insulin. Hepatocytes were seeded on fibronectin-coated plastic dishes (1.5 μg/cm^2), at a density of 80×10^3 viable cells/cm^2. One hour later the medium was renewed, and after 24 h cells were shifted to serum-free hormone-supplemented medium (10^{-8} *M* of dexamethasone and insulin).

Cytotoxicity Indices

Attachment index was measured in fibronectin-coated microtiter plates using 25×10^3 viable cells/well. After 80 min of incubation the protein attached to wells was measured by the method of Lowry et al. (1951). Intracellular lactate dehydrogenase (LDH) was measured after variable exposure time to the drugs. Cells were homogenated in 50 m*M* phosphate buffer (pH 7.5), and the enzymatic activity was measured as described (Bergmeyer and Bernt, 1974).

Biochemical Assays

Glycogen synthesis was evaluated after glycogen depletion with 10^{-7} *M* glucagon, subsequent exposure of cells to the opioids in the presence of 30 m*M* glucose, and evaluation of glycogen content 6 h later. Intracellular glycogen content was estimated after extraction of the polysaccharide (Good et al., 1933) and reaction with anthrone (Hassid and Abraham, 1957). The albumin synthesized and excreted by hepatocytes was measured in culture medium by an ELISA. Aliquots of medium were taken during 4 h and processed as described (Castell et al., 1985). To measure intracellular glutathione, monolayers were detached in 5% TCA, 2 m*M* EDTA, and glutathione was evaluated in the acid supernatant according to the method of Hissin and Hilf (1976).

All experimental assays were carried out in three to four plates per point in four different cell cultures.

RESULTS

Cytotoxic Effects of Opioids on Human Cultured Hepatocytes

The cytotoxicity of morphine, heroin, and meperidine to human hepatocytes, evaluated as impairment of cell attachment index [(attached protein per well in cultures exposed to drugs)/(attached protein per well in controls)], was similar for the three opiates and clearly significant at 1 m*M* (60–70% attachment). On the other hand, methadone was about 10 times more toxic, and at 0.5 m*M* cell attachment was totally inhibited (Fig. 1).

Similar results were obtained when intracellular LDH was measured

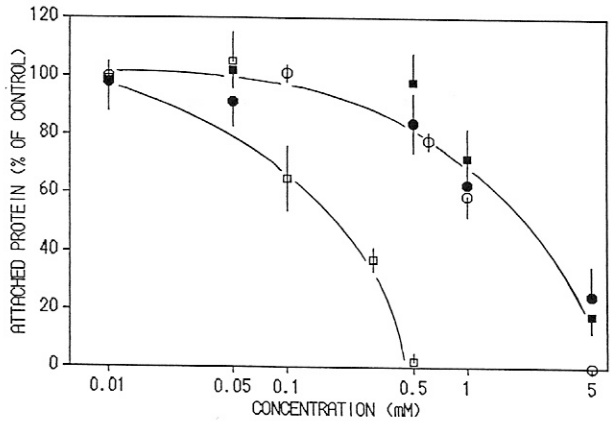

FIGURE 1. Attachment efficiency after 80 min of exposure to opioids. Morphine (●), heroin (■), meperidine (○), and methadone (□) were added to the cultures at the time of plating. Values are the mean of quadruplicate samples for a representative experiment. Bars denote SD, $n = 4$. Best curve fitting (hyperbolic) was calculated by a linear least-squares fit procedure; $r > .95$.

after incubating hepatocytes with increasing amounts of the opiates during the first 24 h. While no appreciable LDH leakage was observed in cells incubated with 0.5 m*M* of morphine, heroin, or meperidine, incubation of human hepatocytes with 0.01 m*M* methadone decreased intracellular LDH significantly and 0.5 m*M* methadone resulted in a complete loss of intracellular LDH (Fig. 2).

Cell survival was also evaluated in cultures after 24, 48, and 72 h of

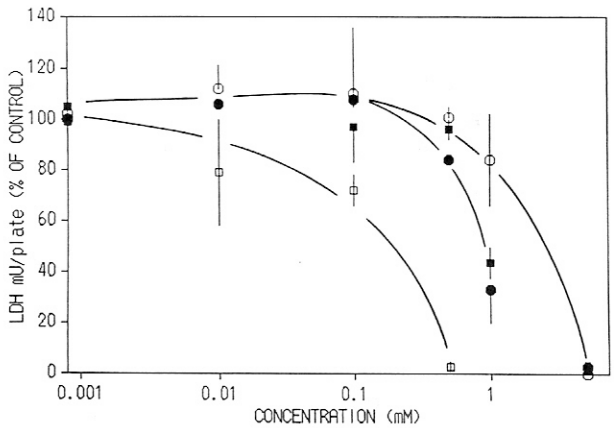

FIGURE 2. Intracellular LDH in cultured hepatocytes after 24 h of exposure to opioids. The opioids were added to cultures 1 h after cell plating. Control cultures had 239 ± 9 mU LDH/plate and 466 ± 64 μg cell protein/plate (3.5 cm/plates). Morphine (●), heroin (■), meperidine (○), and methadone (□). Values are the mean of triplicate samples for a representative experiment. Bars denote SD, $n = 3$. Best curve fitting (hyperbolic) was calculated by a linear least squares fit procedure; $r > .92$.

continuous exposure to the opioids. The drugs were added after the first 24 h of culture and every day at the time of medium renewal. Cellular protein and intracellular LDH losses increased with exposure time. Concentrations of morphine and heroin in the range 0.1–0.5 m*M*, 0.05 m*M* meperidine, and 0.001 m*M* methadone that had no effect after 24 h produced massive effects after 72 h of treatment (40, 35, 50, and 40%, respectively).

Metabolic Effects of Opioids on Cultured Human Hepatocytes

Twenty-four-hour cultures were subjected to variable-term exposure to a wide range of concentrations of the opioids.

A frequent clinical observation after large opiate doses is the evaluation of serum glucose. To investigate whether opiates have a direct effect on hydrolysis of hepatic glycogen, we measured the intracellular glycogen content of human hepatocytes after incubation with increasing doses of opiates. Unstimulated, non-hormone-treated human hepatocytes have an average glycogen content of 1250 ± 177 nmol glucose/mg protein (mean ± SEM, $n = 5$) as determined in 10 different donors. Morphine, heroin, and meperidine caused a dose-dependent mobilization of glycogen with half-maximal effect at 1 m*M* (Fig. 3*a*). The glycogenolytic effect of methadone was more pronounced, and after 24 h of incubation with 0.2 m*M*, about 70% of glycogen had been hydrolyzed in absence of glucagon. In addition, a concentration of 1 m*M* for morphine, heroin, and meperidine and of 0.2 m*M* methadone produced a significant inhibition (40, 30, 40, and 40%, respectively) on glycogen synthesis from 30 m*M* glucose. Concentrations below 0.1 m*M* morphine, heroin, and meperidine and 0.05 m*M* methadone produced no significant effects (Fig. 3*b*).

The effect of opiates on albumin plasma synthesis by human hepatocytes was studied after incubating cells with increasing amounts of the opioids for 24 h and then measuring the albumin synthesis for 4 h. Morphine, heroin, and meperidine had no effect at 0.05 m*M*, but reduced albumin synthesis by 50% in the range 0.4–0.8 m*M* (Fig. 4). The inhibition of albumin synthesis caused by methadone was about 100-fold greater, and 0.005 m*M* caused a 40% inhibition. Exposure of hepatocytes to concentrations below 0.05 m*M* morphine, heroin, and meperidine and below 0.001 m*M* methadone had no significant effects on the albumin synthesis rate.

Maximal depletion of intracellular reduced GSH content was obtained upon incubation of hepatocytes with morphine and heroin for up to 3 h, and methadone and meperidine for up to 6 h. Concentrations of 2 m*M* morphine and 1 m*M* heroin reduced intracellular GSH levels of human hepatocytes to 50% of that of nontreated cells within 2–3 h. On the other hand, 1 m*M* meperidine and 0.2 m*M* methadone produced only a reduction of about 30% after 6 h of incubation (Fig. 5).

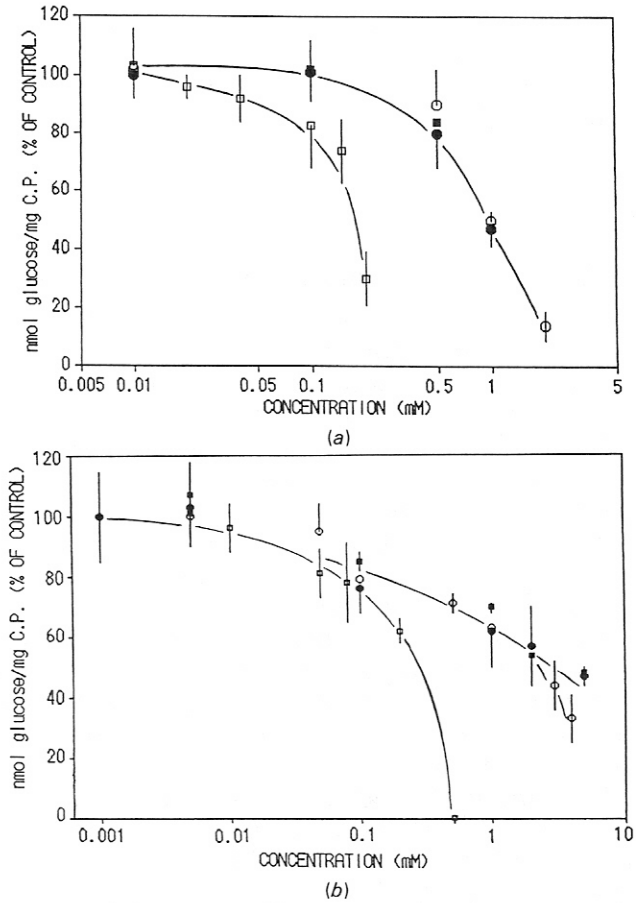

FIGURE 3. Glycogen metabolism in 48-h-old cultured hepatocytes exposed to opioids. (*a*) Glycogenolysis determined after 24 h of exposure to the opioids. Glycogen content in control cells was 1250.5 ± 177.7 nmol glucose/mg cell protein. (*b*) Glycogen synthesis. After glycogen depletion with 10^{-7} *M* glucagon, the cells were exposed to opioids in presence of 30 m*M* glucose, and the glycogen content was determined 6 h later. Control cultures had 85 ± 14 nmol glucose/mg cell protein after depletion and 190 ± 30 nmol glucose/mg cell protein after synthesis. Morphine (●), heroin (■), meperidine (○), and methadone (□). Values are the mean of triplicate samples for a representative experiment. Bars denote SD, $n = 3$. Best curve fitting (hyperbolic) was calculated by a linear least-squares fit procedure; $r > .97$.

DISCUSSION AND CONCLUSIONS

Our study was aimed to investigate whether morphine, heroin, meperidine, and methadone had intrinsic hepatotoxic effects to human hepatocytes and to compare their toxic concentration in vitro with the concentration found in plasma after therapy or self administration by addicts. Human hepatocytes in primary culture obtained from 22 biopsies and cultured under chemically defined conditions were used as experimental approach to investigate toxicity to human liver (Castell

and Gómez-Lechón, 1987; Castell et al., 1988; Gómez-Lechón et al., 1988).

Cell attachment, LDH leakage, and cell survival experiments consistently indicated that cytotoxicity of methadone was about 10 times greater than that of morphine, heroin, or meperidine. A similar picture emerges from the comparison of the metabolic parameters. Methadone inhibited albumin synthesis at concentrations much lower than that of the other three opioids. To better interpret the in vitro results, representative pharmacokinetic data, plasma concentrations after therapeutic doses to elicit analgesia in humans, and plasma levels after doses of opioids administered during tolerance or abuse have been compared with the concentration of drug causing impairment of hepatic cellular function (Table 1).

Appreciable interferences on cell attachment, cellular protein loss, or LDH leakage are only produced at concentrations far larger than those to be expected after therapeutical or even drug abuse dosage (Figs. 1 and 2, Table 1). Cellular protein loss, impariment of monolayer formation, and massive LDH leakage were found at concentrations greater than 1 m*M* for morphine, heroin, and meperidine, while methadone at 0.1 m*M* produced a significant effect. After 72 h of exposure to the opioids, cell survival was affected by lower concentrations of the drug, but still larger than that observed in plasma. LDH leakage, however, is detected at a methadone concentration close to that found in plasma after tolerance doses.

When impairment of metabolic functions of hepatocytes was analyzed, two important features became evident. First, opiates stimulate

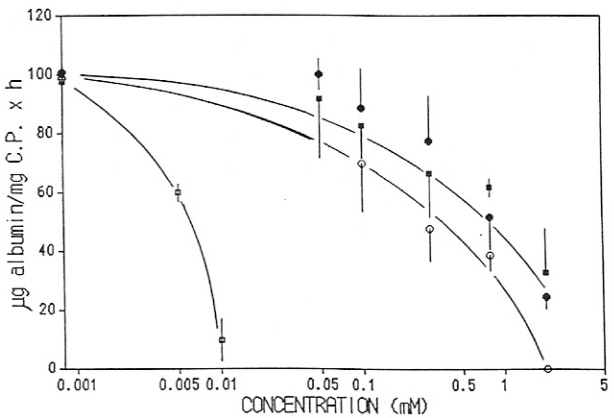

FIGURE 4. Albumin synthesis in 48-h-old cultured hepatocytes determined after 24 h of exposure to the opioids; 100% values correspond to 1.5 ± 0.2 µg/mg cell protein·h. Morphine (●), heroin (■), meperidine (○), and methadone (□). Values are the mean of triplicate samples for a representative experiment. Values are expressed as the mean ± SD, $n = 3$. Best curve fitting (hyperbolic) was calculated by a linear least-squares fit procedure; $r > 0.97$.

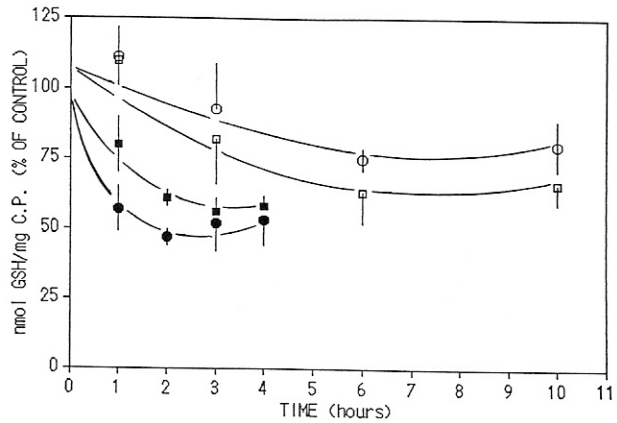

FIGURE 5. Kinetics of intracellular glutathione depletion in 24-h-old cultured hepatocytes incubated with opioids. Glutathione content in control cells was 14.8 ± 2.1 nmol/mg cell protein, at the beginning of the experiment. After adding 2 m*M* morphine (●), 1 m*M* heroin (■), 1 m*M* meperidine (○), and 0.2 m*M* methadone (□), plates were taken at different times and GSH was measured. Values are the mean of triplicate samples for a representative experiment. Bars denote SD, $n = 3$. Best curve fitting (polynomial, degree 2) was calculated by a linear least-squares fit procedure; $r = 0.93$.

glycogenolysis in absence of hormones, methadone being 10 times more active than the other 3 drugs. Similarly, synthesis of glycogen was more strongly inhibited by methadone at the same molar concentration. Morphine-induced hyperglycemia, a phenomenon observed during overdosages, was related to a central nervous system-mediated stimulation of adrenal catecholamines and their subsequent effect on the liver (Feldberg and Shaligram, 1972). Although recently it was indicated that the hyperglycemic action of morphine could be, at least in part, the result of increased glucagon secretion from the pancreas (Ipp et al., 1980), our results clearly show that a direct effect of the opioids on glycogen catabolism is possible at concentrations close to the peak plasma concentration after tolerance dosages. Second, albumin synthesis was the most sensitive parameter. Interestingly, it was possible to observe adverse effects on albumin synthesis at concentrations where no cytotoxicity was evident.

Morphine and heroin undergo microsomal *N*-demethylation and *N*-oxidation by the liver, followed by the formation of glutathione conjugates representing the main detoxification pathways of opioids in rat and mouse hepatocytes (Eklöw-Låstbom et al., 1986; Nagamatsu et al., 1986). The results of our experiments showing a rapid and substantial decrease in intracellular GSH content of human hepatocytes after incubation with either morphine or heroin (Fig. 5) also suggest a similar mechanism for human hepatocytes. Longer exposure was needed for methadone and meperidine to produce some effect, indicating a differ-

TABLE 1. Comparison between the Opioids' Toxic Concentration in Vitro with the Concentration Found in Plasma after Therapeutical or Self Administration by Addicts

Opiate	V_d (1/kg)	$t_{1/2}$ (h)	Dose[a]	Plasma concentration	Lower concentration at which in vitro effect is observed
Morphine	2–4 (1)	3–4 (1)	2.5–20 mg therapy (3) until 4 g tolerance and abuse (4)	4×10^{-5}–3×10^{-4} m*M* 0.06 m*M*	0.1 m*M*: glycogen and albumin synthesis
Heroin	25 (7)	2–3 (1)	1–8 mg therapy (5) until 2 g tolerance and abuse (3)	2×10^{-6}– 10^{-5} m*M* 0.003 m*M*	0.1 m*M*: glycogen and albumin synthesis
Meperidine	4–5 (1)	2–4 (1)	50–150 mg therapy (3) until 3–4 g tolerance and abuse (5)	6×10^{-4}–2×10^{-3} m*M* 0.05 m*M*	0.1 m*M*: glycogen and albumin synthesis
Methadone	3–4 (1)	13–58 (2)	2.5–10 mg therapy (3) until 400 mg tolerance and abuse (6)	3×10^{-5}– 10^{-4} m*M* 0.004 m*M*	0.005 m*M*: albumin synthesis

[a]References: (1) Benet and Sheiner (1985); (2) Sawe (1986), Inturrisi et al. (1987); (3) McEvoy (1987); (4) Williams and Oberst (1946); (5) Jaffe and Martin (1985); (6) data from Spanish Red Cross.

ent pattern of conjugation for meperidine and methadone in human liver.

The toxicity of methadone deserves attention, since this drug is used in the treatment of heroin addicts at relatively high doses and for prolonged periods. Methadone was in all tests the most toxic of all opioids assayed. In addition to its effect on glycogenolysis, it strongly inhibited albumin synthesis, as had been previously observed in hepatoma cells (Beverley et al., 1984).

The results of our work show that therapeutic dosis of the opioids are unlikely to produce irreversible damage to human hepatocytes. However, methadone dosis during tolerance or abuse represents a probable cause of liver dysfunction and may have clinical significance in patients under prolonged treatments with opiates or when tolerance or addiction develops.

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