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Mechanisms Behind the Inhibitory Effect of Ethanol on the Conjugation of Morphine in Rat Hepatocytes[1]

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ABSTRACT

Liver microsomes were isolated by calcium aggregation, and isolated hepatocytes from male Wistar rats were prepared according to a two-step Ca^{++}-free collagenase perfusion method. With the hepatocytes maximal inhibition of glucuronidation (about 40%) was reached at 10 mM ethanol after incubation at 37°C for 60 min. UDP-glucuronic acid concentration and energy charge in the hepatocytes also did decrease maximally (about 90 and 50%, respectively) and the amount of UDP-glucose was tripled in the presence of 10 mM and higher concentrations of ethanol. The alcohol dehydrogenase inhibitor 4-methylpyrazole abolished ethanol-induced inhibition of morphine glucuronidation in the hepatocytes. Acetaldehyde (250–50 μM) and the pH decrease induced by ethanol did not reduce morphine-3-glucuronide formation by the cells. Cellular uptake of morphine and excretion of morphine metabolites were similar in the absence and presence of ethanol. Ethanol (60 mM) did not affect the glucuronidation of morphine (1.7 mM added) during a 30-min incubation at 37°C with the microsomes (UDP-glucuronic acid, 5 mM). When the concentration of UDP-glucuronic acid in the microsomes was lowered from 1 to 0.1 mM, the decrease in morphine-3-glucuronide formation was similar to that observed in cells. The data indicate that the inhibition by ethanol of morphine glucuronidation was due to decreased levels of UDP-glucuronic acid. The mechanism is likely to be inhibition of UDP-glucose dehydrogenase activity by ethanol from increased intracellular NADH/NAD ratio accompanying ethanol oxidation.

Morphine and norcodeine are major metabolites of codeine in perfused liver and isolated hepatocytes from rats (Bodd *et al.*, 1986; Christensen *et al.*, 1984). It has been shown that morphine accumulated to a greater extent in the medium when codeine was incubated with isolated hepatocytes in the presence of ethanol (Bodd *et al.*, 1986). The main metabolic pathway in mammalians for the elimination of morphine is conjugation by glucuronidation. This process appeared inhibited by ethanol in isolated rat hepatocytes (Bodd *et al.*, 1986).

Moldeus *et al.* (1978) observed that ethanol inhibited the glucuronidation of several other compounds in hepatocytes. They suggested that the inhibitory effect of ethanol was mediated by decreased synthesis of UDPGA. However, the level of this intermediate could not be determined. The mechanism was suggested to be inhibition of the UDP-glucose dehydrogenase reaction by the increased NADH/NAD ratio resulting from alcohol dehydrogenase-dependent oxidation of ethanol. The present study was designed to identify the mechanism(s) underlying the inhibitory effect of ethanol on morphine glucuronidation.

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Materials and Methods

Chemicals. Codeine, norcodeine and 3-O-methylnalorphine were gifts from Weiders Farmasøytiske A/S (Oslo, Norway). Morphine and pentobarbital were purchased from Norsk Medisinaldepot (Oslo, Norway). M3G, cyanamide, all nucleotides and their derivatives, β-alanine, *n*-capronic acid, collagenase (type 1), albumin (fraction V defatted) and HEPES buffer were purchased from Sigma Chemical Co. (St. Louis, MO). Other chemicals were purchased as follows: triethanolamine (Merck, Federal Republic of Germany); hydroxypropylmethylcellulose (Dow Chemical Company, Midland, MI); (N-methyl-^{3}H)-morphine (New England Nuclear, Boston, MA); 4-MP (Labkemi, Gothenburg, Sweden); sodium dodecyl sulfate was of chromatography grade (Regis Chemicals, Morton Grove, IL) and acetonitrile was HPLC grade.

Animals. Male Wistar rats (170–340 g) obtained from Møllegaard Hansens Avlslaboratorier A/S (Ejby, Denmark) were kept on a 12-hr light/dark cycle. The rats were deprived of food with free access to water 24 hr before the preparation of microsomes and liver cells.

Studies with liver cells. Liver cells were prepared and diluted in a medium usually containing 5.5 mM glucose, 20 mM HEPES buffer and 2 mM Ca^{++} as described elsewhere (Mørland *et al.*, 1979). The final concentration was 2.0×10^6 cells/ml. Aliquots of 25 ml of cell suspensions were incubated at 37°C under air in 250-ml glass flasks that were shaken continuously at 150 oscillations/min. Cyanamide (0.25 mM) and 4-MP (0.5 mM) were added 10 min before and ethanol (usual concentration 60 mM unless specified otherwise) and acetaldehyde (0.25 mM) were added to the hepatocytes 5 min before morphine (20

ABBREVIATIONS: UDPGA, UDP-glucuronic acid; M3G, morphine-3-glucuronide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; 4-MP, 4-methylpyrazole; HPLC, high-performance liquid chromatography.

μM). Total incubation time after morphine addition to the cell suspensions was 60 min, and samples were taken at the times indicated in the figures and tables. The samples were centrifuged immediately at 1600 × g for 45 sec at room temperature and the supernatants and pellets were frozen separately in liquid nitrogen before they were stored at −20°C.

In an attempt to prevent the extracellular pH decline observed when ethanol concentration exceeded 10 mM (Mørland *et al.*, 1980), three experiments were performed with 100 mM HEPES buffer in the medium. In separate experiments suspensions were made without glucose in the medium.

The uptake of morphine was studied with hepatocytes (6×10^6 cells/ml) incubated with 10 μM morphine (^{3}H-labeled) in the absence and presence of ethanol. The experiments were performed on ice and at 37°C and the cells were separated from the medium as modified from Nilsson and Berg (1977), using an oil phase (100 μl) consisting of dionyl phtalate and dibutyl phtalate (1/3; v/v). Samples (250 μl) were taken at 1, 2, 5, 7, 10, 15 and 25 min after the addition of morphine, which was added 5 min after ethanol. The pellet and the supernatant (100 μl) were dissolved for 18 hr in 1 ml of 2% sodium dodecyl sulfate in counting vials before the measurement of radioactivity (corrected for amount of protein) in a liquid scintillation counter (Rackbeta 1215, LKB, Bromma, Sweden). The small amount of radioactivity found on the cells after incubation (binding) at 0°C was subtracted from the cellular radioactivity found at 37°C to obtain a measure of the cellular uptake of morphine. The control cells that had been incubated for 25 min with tritiated morphine in the absence of ethanol were thereafter washed twice with a buffer solution at 0°C, divided in half and resuspended immediately in 2 ml of medium at 37°C, containing 1 mM unlabeled morphine and incubated in the absence and presence of ethanol. Samples were taken at 1, 2, 10, 15, 25, 45 and 60 min after the addition of the medium. Further procedures were as described above.

Studies with microsomes. The liver microsomes were prepared by calcium aggregation as described previously (Gadeholt *et al.*, 1982). The incubation mixture at 37°C consisted of: morphine hydrochloride, 1.7 mM; UDPGA, 5.0 mM; $MgCl_2$, 10 mM; Tris HCl, 50 mM, pH 7.7; and microsomal protein 3.0 mg/ml plus 60 mM ethanol, when present. The total volume was 1.5 ml and the test tubes were shaken at about 100 oscillations/min. Samples were taken after 10, 20 and 30 min of incubation. The reaction was terminated by 700 μl of 0.6 M perchloric acid to the solution, followed by a brief centrifugation to remove proteins. Control incubations had no UDPGA and zero time blanks with all reactants were prepared by adding 700 μl of perchloric acid immediately after the addition of microsomal protein. In separate experiments, morphine (1.7 mM) was incubated for 10 min under similar conditions with different concentrations of UDPGA ranging from 0.1 to 1 mM.

In three additional experiments, 0.3 mM M3G was incubated for 90 min in the absence and presence of ethanol with the same reaction medium and following the same procedure as described for morphine, except that morphine and UDPGA were not added.

Analytical procedures. To 1 ml of the clear supernatant from the microsomes or the liver cells was added 2 ml of 0.5 M ammonium sulfate, pH 9.3, to prepare the samples for the extraction of the opiates. Measurements of M3G and the other opiates were done by HPLC after column extraction as described previously (Bodd *et al.*, 1986). Protein was determined as described by Lowry *et al.* (1951) using bovine serum albumin as standard. Acetaldehyde and ethanol were analyzed in media using head space gas chromatography according to the methods of Stowell *et al.* (1981) and Meyer (1978).

The nucleotide content of the liver cells was quantified by capillary isotachophoresis. The extraction with cold perchloric acid-methanol and neutralization was done according to Eriksson (1980), with some modifications. We used 400 μl of the cold perchloric acid-methanol extraction media to each pellet. The extraction media contained 0.5 nmol of nicotinic acid as an internal standard. The analysis was performed on a LKB 2127 Tachophor (LKB) with UV detection at 254 nm. The separation occurred in a 40-cm tube at 20°C, except for UDP-glucose that was measured by the use of a 12 cm (inside diameter 0.3 mm) fused silica column (ITABA AB, Järfälla, Sweden) with a column coupling according to Eriksson (1980). The electrolytes were 5 mM HCl plus 0.5% hydroxymethylcellulose plus β-alanine and 10 mM caproic acid at pH 3.89. The following spacer solution was used to increase separation (millimolar): tartaric acid, 0.4; fumaric acid, 0.5; chloroacetic acid, 0.4; trichloroacetic acid, 0.3; glycolic acid, 0.3; glyceric acid, 0.3; saccharic acid, 0.3; acetic acid, 0.3; and laevulinic acid, 0.3. Two microliters of this solution were used with 8 μl of electrolyte extract. The peaks were monitored with an LDC 304-50 computing integrator (Laboratory Data Control, Manchester, England). The pellet remaining after nucleotide extraction was washed with cold perchloric acid and DNA was measured as previously described (Hinrichs *et al.*, 1964).

Expression of results. Values from the cell experiments, except for nucleotide data, were corrected for number of viable cells quantified by means of the trypan blue exclusion test. The concentration time points obtained were used to confirm that first-order kinetics were obeyed. The data from the nucleotide measurements were expressed as area units, and corrected for amount of DNA. The energy status of the cell has been expressed as energy charge, which for the given concentrations of ATP, ADP and AMP was calculated according to the equation:

$$1/2 \times \frac{(ADP) + 2(ATP)}{(AMP) + (ADP) + (ATP)}$$

The results are given as means ± S.D. The data were tested for statistical significance using Wilcoxon's test for paired comparisons or Student's *t* test when the number of observations were less than five.

Results

Studies with hepatocytes. When isolated hepatocytes were incubated with morphine and different concentrations of ethanol, ranging from 2 to 100 mM, inhibition of morphine glucuronidation was observed. There was a significant inhibition of conjugation at 10 mM with no further inhibition at higher ethanol concentrations (table 1). There was no significant change of the initial ethanol concentration during the incubations.

The amount of UDPGA measured in the cell pellets from the same experiments also did decrease to its minimum when the concentration of ethanol exceeded 10 mM (table 1). When correcting for DNA content and cellular water, it was calculated

TABLE 1

Effect of ethanol on the formation of M3G, the level of UDPGA and energy charge from morphine in hepatocytes prepared after diethyl ether narcosis

M3G was calculated from the appearance curves and is given as nanomoles per milligram of cell protein × minutes from 20 μM morphine. UDPGA is calculated as the intracellular concentration after correcting for the DNA and water content of the cells (2.0×10^6/ml) after a 60-min incubation with morphine. The energy charge (see "Materials and Methods" for description) is the energy status measured after a 60-min incubation with morphine. Ethanol was added 5 min before morphine and samples were taken at 2, 10, 30 and 60 min after morphine addition. M3G results are means ± S.D. of three experiments, whereas the data on UDPGA and energy charge are from one of these experiments.

Ethanol Added	M3G Formed	UDPGA	Energy Charge
mM		*mM*	
0	0.058 ± 0.013	0.9	0.43
2	0.056 ± 0.017	0.6	0.41
10	0.038 ± 0.007*	0.1	0.21
30	0.034 ± 0.010*	0.2	0.21
60	0.031 ± 0.003*	0.1	0.22
100	0.027 ± 0.005*	0.1	0.20

* $P < .05$ compared with control.

that the intracellular concentration of UDPGA in the cells before the addition of morphine or ethanol was approximately 1 mM.

UDP-glucose concentrations in the hepatocytes increased to approximately 300% of the control value in the presence of ethanol higher than 10 mM.

With the present analytical method we were not able to measure UTP. The energy charge of the control hepatocytes after a 60-min incubation with morphine were somewhat lower than described previously (Baur *et al.*, 1975), but there was a distinctly lowered energy charge in the ethanol-treated cells (table 1). The energy charge was similar at all ethanol concentrations higher than 10 mM, giving a reduction of approximately 50%.

When hepatocytes were incubated for 60 min in a medium with glucose as well as without, the formation of M3G was similar and the inhibition seen in the presence of ethanol was not altered by absence of glucose in the medium (fig. 1).

In some experiments we tried to explore the importance of ethanol metabolism, and the presence of 4-MP abolished the inhibitory effect of ethanol (fig. 1). 4-MP did not influence the rate of M3G formation.

We wanted to examine the effect of acetaldehyde on morphine conjugation. In experiments using the aldehyde dehydrogenase inhibitor cyanamide and the alcohol dehydrogenase inhibitor 4-MP, in order to reduce metabolism of added acetaldehyde the rate of M3G formation from morphine was unchanged (data not shown). After addition of acetaldehyde in a concentration of 250 μM in these experiments, the mean concentration in the medium after 65 min was 50 ± 28 μM (metabolic rate, 1.5 ± 0.8 nmol/mg of cell protein × min). Also when only 4-MP was used in the presence of acetaldehyde, thus allowing the further oxidation of acetaldehyde, no significant effect was seen on morphine glucuronidation and no acetaldehyde was found in the medium from 7 min onward.

In experiments with the standard 20 mM HEPES buffer, the medium pH was significantly lower (0.033 U) in incubations with ethanol, compared to controls. The extracellular pH was, however, not significantly different in the ethanol-treated cells and the controls in experiments with 100 mM HEPES buffer. Ethanol inhibition of morphine glucuronidation was approximately 50% and similar in experiments with the two different buffer concentrations.

It has been shown previously that morphine accumulates in the hepatocyte, and that the uptake of morphine into the liver cell is energy-dependent (Iwamoto *et al.*, 1978). A possible explanation for our results could have been that cellular uptake of morphine was decreased, or the excretion of M3G was decreased in the presence of ethanol. The uptake of radioactive morphine reached steady state after about 10 min of incubation at 37°C. After pulsing and washing of the cells, the appearance of radioactivity in the medium increased rapidly the first 15 min, thereafter only slowly. No significant differences were observed between the controls and the ethanol-treated cells with regard to uptake and release of radioactivity (data not shown).

Diethyl ether in contrast to pentobarbital has been shown to lower the UDPGA level of the liver cells during anesthesia (Eriksson and Stråth, 1981; Christensson and Eriksson, 1985). In an experiment using cells from an animal given pentobarbital as the anesthetic the magnitude of ethanol inhibition was 60% and within the same range as observed with diethyl ether (see table 1).

Studies with microsomes. When morphine was incubated with the microsomes, the formation of M3G was linear with time. There was no significant difference between the rate of M3G formation in the absence (0.91 ± 0.24 nmol/mg of protein × min) and presence (0.84 ± 0.26 nmol/mg of protein × min) of ethanol. When the concentration of UDPGA was lowered from 1 mM (which corresponds to the level found in control cells) to 0.1 mM (which corresponds to the level found in cells after a 60-min incubation with 60 mM ethanol) a decrease of M3G formation of about 60% was observed (fig. 2). No glucuronidase activity was observed when M3G was incubated with the microsomal fraction for 90 min (data not shown).

Discussion

Moldeus *et al.* (1978) observed the ethanol effect on glucuronidation in hepatocytes that were isolated from phenobarbi-

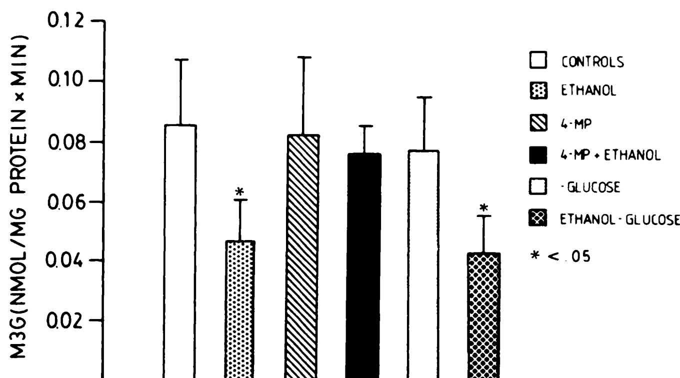

Fig. 1. Formation of M3G after morphine incubation with hepatocytes, ethanol (60 mM) effect in the absence and presence of 4-MP (0.5 mM) and glucose. The control cells (2.1 × 10^6/ml) were incubated with morphine (20 μM) for 60 min with the usual presence of glucose (5.5 mM) in the medium. The formation of M3G has been expressed as nanomoles per milligram of protein × minutes and represents means + S.D. of three experiments.

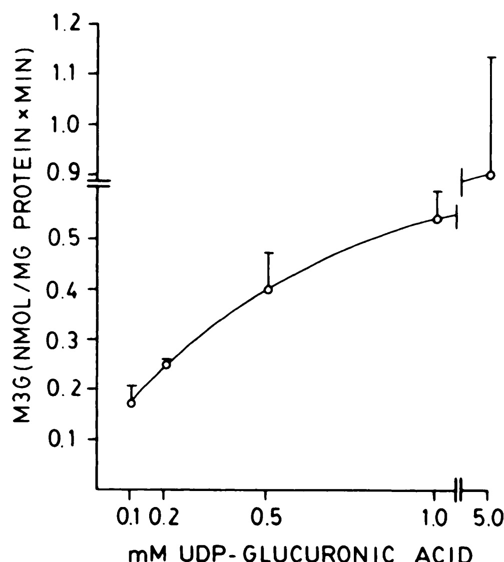

Fig. 2. Glucuronidation of morphine in microsomes in the presence of different concentrations of UDPGA. The microsomes (3 mg of protein per ml) were incubated with morphine (1.7 mM) in the presence of UDPGA for 10 min. The rate of M3G formation has been expressed as nanomoles per milligram of protein × minutes and represents means + S.D. of three experiments.

tal-treated rats. We observed an analogous phenomenon in hepatocytes from noninduced rats.

When microsomes were subjected to UDPGA variations within the range seen in the hepatocytes after ethanol, about the same decrease in the rate of M3G formation was observed as in the cells. This is consistent with the conjugation of *p*-nitrophenol which correlates with the UDPGA content in the livers (Reinke *et al.*, 1981). It indicates a possible role of the decreased UDPGA concentration as mediator of the ethanol effect on the conjugation process. This is supported further by our finding that in the presence of sufficient cofactors ethanol did not inhibit microsomal 3-glucuronidation of morphine.

The data suggest that inhibition of the UDP-glucose dehydrogenase reaction might be a mechanism by which ethanol exerts its effect, as an increased UDP-glucose concentration was found when the level of UDPGA decreased. A possible cause for the inhibition might be the increased NADH/NAD ratio caused by ethanol oxidation because UDPGA formation from UDP-glucose is inhibited by NADH (Dutton, 1966). *p*-Nitrophenol conjugation in phenobarbital-treated rats was inhibited by compounds which increased the NADH/NAD ratio (Reinke *et al.*, 1981) and several of our findings support this notion. First, the inhibitory effect of ethanol reached maximum at 10 mM, a concentration at which alcohol dehydrogenase is already saturated (Higgins, 1979) and second, the alcohol dehydrogenase inhibitor 4-MP did abolish the ethanol effect on morphine glucuronidation. This indicates that alcohol and/or aldehyde dehydrogenase-dependent oxidation possibly plays a role in the inhibition observed.

Acetaldehyde in a concentration higher than 50 μM, or acetaldehyde oxidation *via* aldehyde dehydrogenase did not alter the rate of morphine conjugation, excluding that either direct or metabolic effects of acetaldehyde mediated the ethanol effect. The concentration of acetaldehyde is usually less than 10 μM in isolated hepatocytes metabolizing ethanol at *in vivo* rates (Veech, 1981).

Because both glucuronidation and UDP-glucose oxidation are pH dependent (Dutton, 1966), the possibility existed that the formation of H^+ ions caused by ethanol and acetaldehyde oxidation could depress the rate of morphine glucuronidation. This was ruled out because the inhibitory effect of ethanol was unchanged when pH was kept constant.

Glucose enters the glucuronidation pathway after biotransformation to glucose-1-phosphate, UDP-glucose and UDPGA. UTP is required for the formation of UDP-glucose, but normally cellular energetics do not limit glucuronidation (Thurman and Kauffman, 1980). Increased gluconeogenesis from pyruvate and the energy consuming carbon recycling reactions might be the reasons for the depressed energy charge in the presence of ethanol (Cederbaum and Dicker, 1981; Meijer *et al.*, 1975). It may be calculated from the data of Veech *et al.* (1972) that acute ethanol administration in a concentration less than 10 mM depressed the energy charge by approximately 10% in freeze-clamped livers from rats which had been fasted for 48 hr. In our study, the lower energy status (50%) in the presence of ethanol was not rate limiting for glucuronidation, as a higher concentration of UDP-glucose was measured in ethanol experiments.

In summary, the glucuronidation of morphine was inhibited in the presence of ethanol in hepatocytes. We also found reduced synthesis of UDPGA as a result of inhibition at the level of the UDP-glucose dehydrogenase reaction.

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