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COVALENT BINDING OF MORPHINE TO ISOLATED RAT HEPATOCYTES

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Abstract—Incubation of [^{3}H]morphine with isolated hepatocytes caused covalent binding of [^{3}H]-morphine to hepatocellular proteins. Sulfhydryl compounds protected against morphine-induced toxicity and decreased covalent binding. Analysis of covalently bound proteins in the cytosol by electrophoresis indicated that covalently bound radiolabel was associated with macromolecules greater than 25 kDa and increased throughout the incubation. In contrast, covalent binding to the particulate fraction was highly selectively associated with three protein bands of 50–53 and 33 kDa. Covalent binding of morphine to particulate fraction proteins was observed in hepatocytes which exhibited cellular damage. We conclude that the covalent binding of morphine to protein is associated with morphine-induced cytotoxicity.

Many foreign compounds undergo biological transformation, which involves the formation of highly reactive, metabolic intermediates. These reactive intermediates may then form covalent bonds with cellular macromolecules, reactions which have been suggested to be responsible for the toxicity of many chemicals.

Morphine is a widely used analgesic. However, large overdoses of morphine cause liver dysfunction in experimental animals [1–3]. This toxicity seems to be caused by a direct effect of morphine on the liver, because the incubation of isolated rat hepatocytes with morphine results in glutathione (GSH†) depletion followed by loss of cell viability [4, 5].

Morphine was converted to an electrophilic intermediate, morphinone, by morphine 6-dehydrogenase [6], and morphinone was trapped by conjugation with endogenous GSH, forming 8*S*-(glutathion-*S*-yl)dihydromorphinone, which was excreted into bile [7]. Adduct formation of morphine (or its metabolites) with acid-insoluble tissue materials has been reported [8–10]. Mechanistic studies have linked morphine-induced hepatotoxicity to the formation of an electrophilic metabolite that binds covalently to macromolecules [3, 10, 11]. To understand better the significance of such binding for the initiation and progression of morphine hepatotoxicity, it is important to identify the macromolecules to which the electrophilic metabolite binds.

The object of this study was to gain further insight into the mechanisms of morphine-induced hepatotoxicity and the extent and macromolecular specificity (with respect to molecular mass) of the covalent binding of morphine-derived radioactivity to hepatic macromolecules.

MATERIALS AND METHODS

Chemicals. [^{3}H]Morphine (*N*-methyl[^{3}H], 50.6 Ci/mmol, radiochemical purity 99%) and Aquasol-2 premixed organic scintillation mixture were obtained from New England Nuclear (Boston, MA, U.S.A.). Soluen−350 tissue solubilizer was obtained from Packard Instrument Co. Collagenase was purchased from Boehringer Mannheim (Mannheim, F.R.G.). Electrophoresis chemicals and premixed standard were from the Sigma Chemical Co. (St Louis, MO, U.S.A.). The fluorographic reagent Amplify was from Amersham International (Amersham, U.K.). X-ray film (X-Omart AR-5) was from Kodak. All other solvents and reagents were of the best grade commercially available.

Animals and treatments. Male Sprague–Dawley rats (230–250 g) purchased from SLC (Shizuoka, Japan) were housed in stainless-steel wire cages and allowed to acclimatize for at least 2 weeks before use. Hepatocytes were isolated as described by Moldéus *et al.* [12] and used immediately. The viability of the hepatocytes, determined by Trypan blue exclusion, was 92–97%.

Cell viability and GSH determination. Cellular viability was estimated from the activity of lactate dehydrogenase that leaked from damaged cells [12]. The intracellular level of GSH was estimated by the fluorometric method of Hissin and Hilf [13] or by the HPLC method of Reed *et al.* [14].

Covalent binding of [^{3}H]*morphine to isolated hepatocytes.* The covalent binding of [^{3}H]morphine to the macromolecules of hepatocytes was also determined. Hepatocytes (10^6 cells/mL) were suspended in Krebs–Henseleit buffer supplemented with 12.5 mM 4-(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid (pH 7.4) and incubated with [^{3}H]morphine (0.5 mM), sp. act. 5.4 mCi/mmol, at 37°. After various amounts of time, the reaction was terminated by removing the hepatocytes from the incubation mixture by centrifugation at 600 *g* for 3 min, then 0.5 M methanolic hydrochloric acid was added to the cell pellets. After intensive mixing they were filtered through a Whatman GF/B filter. The pellets on the filters were washed exhaustively with methanol, solubilized with 0.5 mL of Soluen-350, and 10 mL of scintillation fluid (Aquasol-2) was

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† Abbreviations: GSH, glutathione; SDS, sodium dodecyl sulphate.

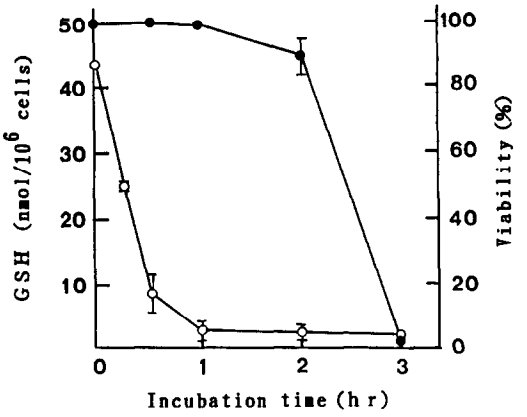

Fig. 1. Effects of morphine on intracellular GSH level and viability of hepatocytes. GSH concentration and lactate dehydrogenase leakage from injured cells were measured in hepatocyte suspensions incubated in the presence of 0.5 mM morphine. GSH level and viability at 3 hourly time points in control cells without morphine were 38 ± 2 and 92 ± 5, respectively. Data represent means ± SD of three experiments. (○) GSH concentration, (●) cell viability.

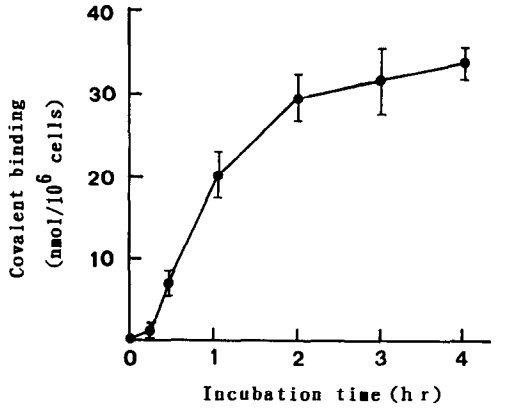

Fig. 2. Covalent binding of radioactivity following incubation of hepatocytes with [^{3}H]morphine. Isolated rat hepatocytes (10^6 cells/mL) were incubated with 0.5 mM [^{3}H]morphine at 37°. After varying amounts of time, total covalent binding of the radioactivity to acid-insoluble materials was measured. Data represents means ± SD of three experiments.

added. The radioactivity of bound [^{3}H]morphine was measured using a liquid scintillation spectrometer (Aloka, LSC-673) after diminishing chemical luminescence. In some experiments, hepatocytes were preincubated (37°) with various sulfhydryl reagents (1 mM) for 30 min prior to the addition, of [^{3}H]-morphine, and were presented throughout the incubation.

Electrophoretic resolution of morphine–protein conjugates. Hepatocytes (10^6 cells/mL) were incubated at 37° with 0.5 mM [^{3}H]morphine, sp. act. 11 mCi/mmol. After various amounts of time, incubation was terminated by removing the hepatocytes from the incubation mixture by centrifugation at 600 *g* for 3 min. The cells were suspended in 1 mL of 0.15 M KCl and lysed by sonication for 40 sec at 4°. The sample was centrifuged at 800 *g* for 10 min, removing nuclei and cell debris. The supernatant was decanted and centrifuged further at 10,500 *g* for 1 hr at 4° and subcellular fractions (800–105,000 *g* sediment, containing mitochondrial fraction and microsomal fractions, and 105,000 *g* supernatant) were prepared. The proteins from each fraction were first resolved in 2% sodium dodecyl sulphate (SDS) and dialysed against 10 mM of sodium phosphate buffer (pH 7.4) using a Spectrapor-3 dialysis tube. The dialysed samples were electrophoresed on 12% SDS-polyacrylamide slab gels (2 × 120 × 135 mm) according to the method of Laemmli [15]. The gel was then stained with Coomassie brilliant blue R-250 and treated with the fluorographic reagent Amplify. Upon completion of electrophoresis, gels were dried and autoradiographed at −80° for 1–2 weeks.

RESULTS

Effects of morphine on cellular GSH level and cell viability

As shown in Fig. 1, the addition of morphine to the suspension of isolated hepatocytes induced a marked decrease in the level of GSH and reduced cell viability. The GSH level dropped rapidly, reaching less than 10% of the control value after 30 min of incubation. Loss of cell viability was delayed, but almost 100% of the cells were damaged after 3 hr of incubation.

Covalent binding of [^{3}H]morphine to isolated hepatocytes

[^{3}H]Morphine was incubated with hepatocytes and its covalent binding to macromolecules was determined as described in Materials and Methods. A time-dependent increase in the covalent binding of morphine-related radioactivity to macromolecules was observed in hepatocytes isolated from rats (Fig. 2). The covalent binding was delayed until hepatic GSH, which normally conjugates preferentially with the reactive metabolite, was depleted.

Effect of sulfhydryl compounds on morphine toxicity

To clarify whether the morphine-induced cell damage was due to covalent binding to macromolecules, hepatocytes were incubated with sulfhydryl compounds and [^{3}H]morphine. With the addition of *N*-acetylcysteine or GSH to the incubation medium, covalent binding was decreased and no loss of cellular viability as a result of morphine was observed (Table 1). The presence of glycine, glutamate and methionine (GSH biosynthesis precursors) also reduced the covalent binding of morphine to cellular macromolecules and protected against morphine toxicity. On the other hand, the addition of glycine and glutamate did not reduce the covalent binding of morphine or morphine-induced toxicity. Therefore, it appears that sulfhydryl compounds play a protective role against the covalent binding of morphine metabolites to proteins.

Table 1. Effects of GSH precursors on morphine-induce toxicity

Treatment	Covalent binding (nmol/10^6 cells) 1	2	3	4	Viability at 4 hr (%)
Control	14	16	34	38	0
Glycine, glutamate	14	16	29	35	0
Glycine, glutamate, methionine	10	14	15	24	70
GSH	10	12	15	20	70
N-Acetylcysteine	7	13	14	16	90

Isolated rat hepatocytes (10^6 cells/mL) were incubated with 0.5 mM [^{3}H]morphine at 37°. Each compound was added at a concentration of 1 mM 15 min prior to incubation with [^{3}H]morphine. After varying amounts of time, total covalent binding of the radioactivity to acid-insoluble materials was measured. Cell viability was measured by LDH leakage.

Data represent means of two experiments.

Macromolecular mass specificity of morphine binding

The detection of morphine-bound proteins was evaluated in three subcellular fractions from hepatocytes incubated with morphine. A time-dependent increase in the covalent binding of morphine-related radioactivity to macromolecules was observed in rat hepatocytes (Fig. 2). These quantitative differences in binding were also reflected in the amount of radioactivity detected from SDS–PAGE, as indicated by a comparison of Figs 2 and 3B. The resulting autoradiographs demonstrated that the covalent binding of [^{3}H]morphine to proteins was highly selective (Figs 3 and 4); not all protein bands detected by Coomassie brilliant blue bound to [^{3}H]morphine. In the cytosol fraction, several discrete protein bands with an apparent molecular mass greater than 25 kDa were detected by autoradiography (Fig. 3B). In contrast, three major protein bands, 68 kDa, 50–53 kDa and 33 kDa, were detected in the particulate fraction (Fig. 4B). In some cases, protein bands that represented only a small fraction of the total protein on the gel contained relatively large amounts of [^{3}H]morphine.

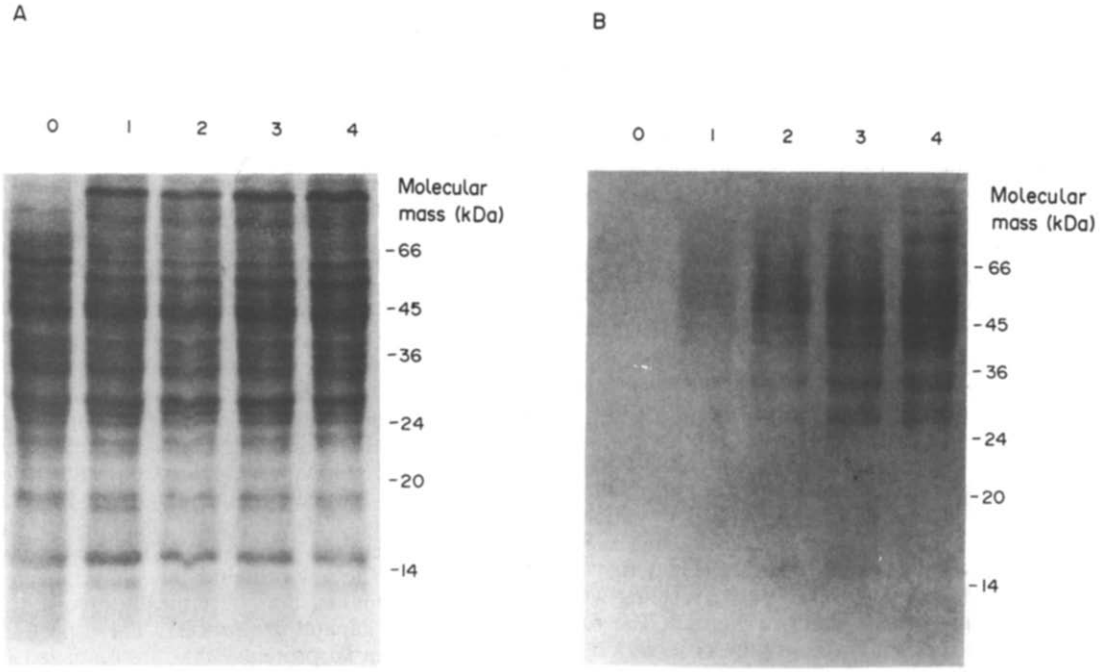

Fig. 3. Electrophoretic pattern of [^{3}H]morphine-bound proteins in cytosol fractions following incubation of hepatocytes with 0.5 mM [^{3}H]morphine. The reaction was terminated at varying times (lane 0, 0 hr; lane 1, 1 hr; lane 2, 2 hr; lane 3, 3 hr; lane 4, 4 hr). All lanes are from 12% SDS–PAGE run under reducing conditions. Profiles of cytosole proteins stained with Coomassie brilliant blue (A) and autoradiographs of [^{3}H]morphine-bound proteins (B).

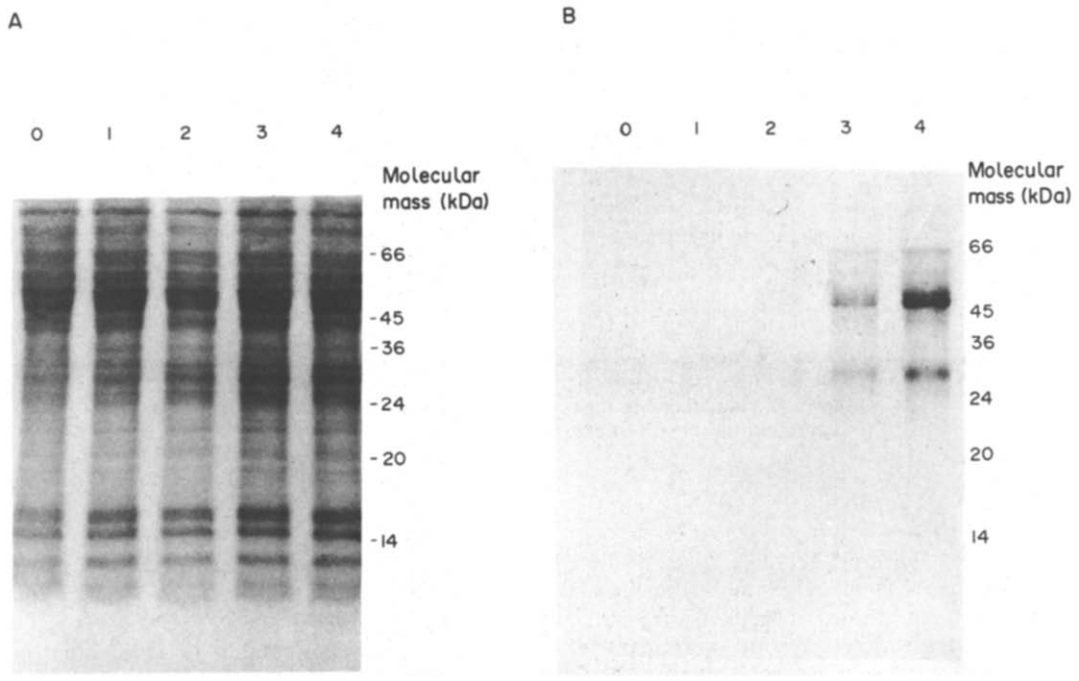

Fig. 4. Electrophoretic pattern of [^{3}H]morphine-bound proteins in the particulate fraction following incubation of hepatocytes with 0.5 mM [^{3}H]morphine. The reaction was terminated at varying times (lane 0, 0 hr; lane 1, 1 hr; lane 2, 2 hr; lane 3, 3 hr; lane 4, 4 hr). All lanes are from 12% SDS–PAGE run under reducing conditions. Profiles of particulate fraction proteins stained with Coomassie brilliant blue (A) and autoradiographs of ^{3}H-morphine-bound proteins (B).

DISCUSSION

Acute morphine administration causes a depletion of GSH in mice [2, 3]. Morphine also decreases GSH levels in isolated rat hepatocytes [4, 5]. In this study, we observed that morphine caused a rapid decrease in GSH in hepatocytes, the covalent binding of its metabolites to cellular macromolecules and cell death (Figs 1 and 2). GSH has been implicated in the maintenance of cellular integrity and is said to play a protective role when cells are attached by nucleophilic chemicals. The addition of sulfhydryl compounds or GSH precursor amino acid decreased the covalent binding of morphine and cell toxicity (Table 1). Thus, sulfhydryl compounds protect hepatocytes against morphine toxicity by virtue of their direct reaction with toxic metabolite via the sulfhydryl group. Morphine was metabolized to the electrophilic intermediate morphinone, which reacted non-enzymatically with GSH by Michel addition [2] or enzymatic reaction [16]. Thus, GSH biosynthesized from precursors protects hepatocytes against morphine toxicity by directly reacting with toxic metabolites via the sulfhydryl group. The role of cellular GSH is supported by the observation of a lag between GSH depletion and covalent binding to macromolecules and lack of cellular viability (Figs 1 and 2). Based on these results, we proposed that the covalent binding of morphine metabolites to macromolecules is linked to the liver toxicity of morphine.

The mechanism of the late stage of cell toxicity remains unclarified, but several ideas have been proposed. They involve covalent binding of the reactive metabolite to essential hepatic proteins [17], oxidation of macromolecules, especially cation pumping ATPase [18], and alteration in the electron transport chain of the cell [19]. Ultrastructural studies have also shown the late stage of liver necrosis to be associated with structural damage to subcellular components, including mitochondria [20].

Electrophoretic separation of [^{3}H]morphine-bound proteins was also studied in subcellular fractions of hepatocytes. Covalent binding was delayed until hepatic GSH, which normally conjugates preferentially with the reactive metabolite, was depleted. Three major protein bands, 68 kDa, 50–53 kDa and 33 kDa, were detected by autoradiography in the particulate fraction 3 to 4 hr after incubation (Fig. 4B). Covalent binding to the particulate fraction is associated with the initiation of cell death. The few protein bands that appeared in morphine binding may be critically involved in mediating the hepatotoxic process. These proteins could be "essential proteins" that are damaged by arylation and initiate cellular perturbations which lead to hepatotoxicity. It is tempting to speculate that reactive morphine metabolites attack mitochondrial membrane proteins, alter the mitochondrial function and perhaps lead to an irreversible final stage in the process of cell necrosis.

The covalent binding of [^{3}H]morphine to cytosolic proteins increased with time. Several discrete protein bands with an apparent molecular mass greater than 25 kDa were detected by autoradiography (Fig. 3B). We have shown previously that morphine is metabolized to morphinone, which then binds covalently to the thiol group of proteins [10]. The depletion of protein thiol groups is not necessarily related to lethal cell injury [21]. Therefore, covalently bound cytosolic proteins may serve a limited function in reducing the concentration of reactive metabolite. Also, covalent binding to the particulate fraction is found only at 3 hr, a time when most cells have lost viability. This could indicate either that the particulate fraction is protected until the cells are dead and covalent binding is a consequence of loss of viability, or else that the cells can maintain viability until covalent binding in the particulate fraction delivers the final blow.

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