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TOXICITY OF HEPARIN IN ISOLATED RAT HEPATOCYTES

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Abstract—The effect of heparin on isolated rat hepatocytes in monolayer culture was assessed to investigate the observed increase in serum aminotransferase activity in patients treated with heparin for thromboembolic disorders. Cells were treated with porcine intestinal mucosal heparin or beef lung heparin in concentrations ranging from 0.01 to 100 units/ml. Toxicity was evaluated based on cell damage or death measured by LDH release into the culture media as a fraction of total system LDH (LDH index). Toxicity appeared at concentrations between 1 and 10 units/ml ($P < 0.05$). The uptake and binding of heparin by the hepatocyte were evaluated by addition of tritium-labeled heparin to the cultures. Sucrose gradient centrifugation with isolation of the liver plasma membranes (LPM) showed little membrane binding of heparin. The majority of intracellular heparin was located in the cytosol fraction. Heparin gains access to hepatocytes and causes a dose-related toxic effect resulting in cell damage and death. This investigation indicates that the increased serum aminotransferase concentrations seen with heparin treatment may be due to a direct hepatotoxic effect of heparin.

Heparin, a tissue extract of polyanionic electrolyte substances of various molecular weights, has been in clinical use for the treatment of thromboembolic disorders for over 30 years. Although heparin is used widely and has been the subject of a multitude of investigations, the heterogeneous molecular mature of the preparation and various animal sources have precluded exact definition of the physiologic and pharmacologic effects. Several adverse effects of heparin including hemorrhage, thrombocytopenia, allergic reactions and osteoporosis have been observed [1]. Recently, there have been reports of elevated serum aminotransferases [aspartate aminotransferase (AST), alanine aminotransferase (ALT)], associated with heparin use [2–6]. In these reports, serum aminotransferase concentrations increased to abnormal values in up to 89% of treated subjects and returned to normal either during treatment or after discontinuation of treatment. The study of Saffle *et al.* [5] was performed on healthy volunteers, thus eliminating the possible confounding effects of pulmonary embolism or myocardial infarction on serum enzymes which may have been present in patient studies. The elevation of ALT was consistently greater than that of AST and suggested hepatocellular damage. Despite this apparent toxic effect, there have been no reports of clinically significant liver injury due to heparin treatment, even during long-term treatment of thromboembolic episodes [7]. The magnitude of the injury thus seems small in the clinical setting. The anticoagulant activity of heparin has precluded liver biopsy as a mechanism for studying cellular damage. This study was undertaken to investigate the possible hepatocellular damage induced by heparin through the use of rat hepatocytes, a useful *in vitro* model for hepatotoxicity [7–11].

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MATERIALS AND METHODS

Monolayer hepatocyte cultures. Hepatocytes were isolated and cultured in monolayer using a modification of methods previously reported [11–13]. Four days following subtotal hepatectomy in adult Sprague–Dawley rats, the regenerated livers were perfused *in situ* with 0.05% crude collagenase in 0.01 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes)-buffered Hanks' salt solution. The washed parenchymal cells were suspended in Eagle's Minimum Essential Medium (MEM) (Gibco Laboratories, Grand Island, NY) with 2.5 mM Hepes buffer and Hanks' salts containing 50 mg/l gentamicin (Schering Pharmaceuticals, Kenilworth, NJ). Cell viability was confirmed by trypan blue exclusion of greater than 90%. Suspensions of 2.5×10^6 cells in 2.5 ml of MEM were placed in 60×15 mm plastic petri dishes and incubated for 24 hr in a humidified chamber at 37° under 95% O_2 and 5% CO_2 to allow formation of monolayers. Following the 24-hr stabilization period, the cells were washed and incubated with fresh medium (control plates) and medium containing 0.01, 0.1, 1.0, 10.0 and 100.0 units/ml of heparin from either beef lung (Upjohn Co., Kalamazoo, MI) or porcine intestinal mucosa (Sigma Chemical Co., St. Louis, MO). The plates were incubated for an additional 4–24 hr following replacement of the media. At the end of this period, the media were withdrawn and passed through 3 μm Millipore filters. The hepatocytes were lysed in 2.5 ml of deionized water, scraped from the plate bottom, and centrifuged at 9000 *g* for 10 min. Both the filtrate and the super-

natant fraction were analyzed for lactate dehydrogenase (LDH) activity [14]. LDH activity has been shown to correlate with cell membrane disruption and is thus regarded as a reliable indicator of cell damage and/or death under the conditions of this study [15]. Toxicity was measured by the LDH index which is the ratio of LDH released into the medium divided by the total LDH in the medium and in the cells, i.e. the percentage of total LDH which is released into the medium. To assure that the LDH index did not reflect a change in total LDH in the system, all data were analyzed to exclude experiments in which cells did not have a normal LDH content at the beginning of the experiment. Each type of heparin was tested in hepatocytes collected from at least three different animals with five plates of cells for control and each heparin concentration.

Heparin uptake studies. Tritium-labeled porcine heparin (New England Nuclear, Boston, MA) (1×10^5 dpm/ml) was added with unlabeled heparin (10 units/ml medium) to stable hepatocytes in culture to demonstrate cellular uptake and/or binding. Following a 24-hr treatment period, the supernatant fraction was collected. Each plate was rinsed twice with saline to remove residual radioactivity not associated with the hepatocytes. The cells were lysed with water (2.5 ml/plate), scraped from the culture dish, and centrifuged at 10,000 *g* for 10 min. The supernatant (cytosol fraction) was collected, and the pellet was resuspended in water and recentrifuged. The resulting supernatant fraction was collected and the pellet was resuspended (cell membranes and debris) in water for scintillation counting (Beckman model L58100, Beckman Instruments, Fullerton, CA).

The influence of protein binding on heparin uptake and binding by the hepatocyte was investigated by the addition of bovine albumin (Sigma Chemical Co.) to the heparin-treated culture medium. The above fractionation procedure was repeated following a 24-hr treatment period with albumin. LDH measurements were conducted concurrently to determine the association of changes in uptake and binding on measured toxicity.

Cell fractionation for the isolation of plasma membranes was conducted to determine the extent of heparin binding to the membrane. Cells were cultured and treated as outlined above. The medium was collected, plates were rinsed twice with saline, and the cells were scraped from the plate and suspended in saline. The cells were concentrated by slow centrifugation for 5 min and then homogenized (50 strokes) in a 7 ml capacity Dounce "A" homogenizer. The homogenate was inspected microscopically to confirm cell disruption and then diluted to 50 ml with 1 mM $NaHCO_3$ before centrifugation at 1500 *g* for 10 min. The supernatant (cytosol fraction) was collected, and the pellet was resuspended in 1 mM $NaHCO_3$ and centrifuged at 4500 *g* for 10 min. The pellet was then suspended in 2 ml of bicarbonate buffer and dispersed in 40 ml of 77% sucrose. Sucrose gradients were constructed with concentrations of 77%, 48% and 40% and centrifuged at 66,000 *g* for 120 min. The interface areas were selectively collected, diluted to 25 ml with bicarbonate buffer, and centrifuged at 15,000 *g* for 10 min. The cellular debris was collected at the 77/48% interface with liver plasma membranes (LPM) located at the 48/40% interface.

Fractions from the LPM isolation procedure were analyzed for LDH activity as detailed above. 5′-Nucleotidase (5′-NT) activity was measured as previously reported [16–19]. Scintillation counting was performed on all aqueous samples using a 0.5 ml aliquot combined with 10 ml BIOFLUOR (New England Nuclear). Quenching analysis was evaluated using a tritium standard (toluene, New England Nuclear) combined with untreated fractionation material. Standard quench curves were developed for correction of cpm to absolute dpm during each analysis. One-way and two-way analyses of variance were used to evaluate differences in mean LDH index between various heparin concentrations and beef versus pork heparin. Student's *t*-test was used to assess the change in toxicity resulting from albumin treatment. Mean values are reported as mean ± standard deviation. The possible interference of heparin with the assay of LDH was tested by addition of various concentrations of heparin to a standard solution of LDH (Sigma Chemical Co.).

RESULTS

Hepatocytes exposed to heparin showed systematic increases in LDH index compared to control plates. Following the 24-hr stabilization period, increased toxicity was seen with lengthening of the duration of exposure to heparin. Exposure to 10 units/ml for a 4-hr period resulted in some increase in LDH index over control (51.7 ± 4.8 vs. $44.7 \pm 3.0\%$, $P < 0.01$), while the 24-hr exposure resulted in greater toxicity (81.2 ± 1 vs. $69.3 \pm 2.0\%$, $P < 0.001$). Due to the increased effect following the 24-hr exposure period (a total of 48 hr in culture), this time period was selected for further experiments.

Hepatocytes from six animals were treated with either beef or pork heparin at concentrations ranging from 0.01 to 100 units/ml. A two-way ANOVA was conducted to evaluate the effects of both heparin source and concentration on the LDH index. No difference was detected between beef and pork heparin. The dose–response relationship between heparin concentration and LDH index is illustrated in Fig. 1. Concentrations of 0.01 and 0.1 units/ml were not toxic and actually produced small decreases in LDH index; however, increased LDH index was evident at the higher concentrations ($P < 0.02$). Furthermore, disruption of the monolayer was seen at this heparin concentration and confirms the toxic effect. Heparin, when added to standard solutions of LDH, did not interfere with the measurement of LDH activity.

Exposure of the cultured hepatocytes to radiolabeled heparin revealed that from 1.4 to 8.3% of the total radioactivity was bound or taken up by the hepatocyte. Distribution of the heparin between subcellular fractions showed no evidence that heparin was concentrated on the plasma membrane (Table 1). Correlation between 5′-NT and radiolabel ranged from −0.11 to 0.43 and was not significant. LDH activity was not detectable in the plasma mem-

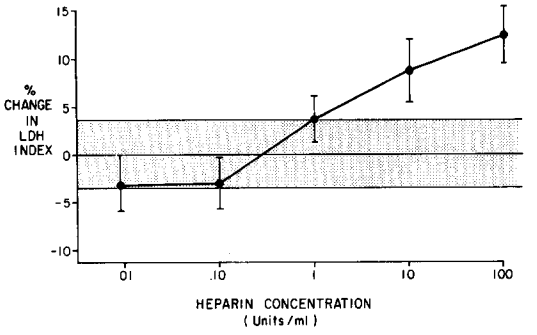

Fig. 1. Dose–response relationship between heparin concentration (units/ml) and mean LDH index (± S.D.) normalized for control LDH index. Cells were exposed to heparin for 24 hr following a 24-hr stabilization period.

Table 1. Distribution of radioactivity and 5′-nucleotidase activity in sucrose gradient fractionation following 24-hr treatment of isolated hepatocytes

Animal No.		Plasma membrane	Cell residue	Cytosol fraction
022	Radiolabel*	4	14	82
	5′-NT†	104	5	55
023–1	Radiolabel	1.2	7.8	91
	5′-NT	20.3	2.6	20.1
023–2	Radiolabel	1.0	60	39
	5′-NT	15.7	16.3	9.93

* Radioactivity is expressed as percent of total associated with cellular fractions.

† 5′-NT = 5′-nucleotidase activity expressed as μmoles phosphorous released per hr per mg protein.

brane or cell residue fractions (activity < 10 I.U./ml). Although there was 5′-NT activity in the cell residue and cytosol fractions, there was adequate enzyme activity in the membrane fraction to allow assessment of the relationship between radiolabel and 5′-NT distributions. These experiments indicated that from 1 to 4% of the cell-associated heparin was bound to the plasma membranes. The remainder of cell-associated heparin was contained in the cytosol fraction or in the residual cell debris. Table 2 shows the change in LDH index and radioactive uptake and binding induced by the addition of albumin to the culture medium. The mean control LDH index did not increase significantly in the albumin-treated cells. However, albumin treatment resulted in significantly less toxicity in the heparin-treated cells ($P < 0.01$). The mean increase in LDH index was 12.4% for the heparin treated cells versus 5.4% for the heparin plus albumin-treated cells.

DISCUSSION

A number of experiments have been conducted to illustrate the interaction of heparin with isolated rat hepatocytes in monolayer culture. The results indicate that the aminotransferase elevations seen in patients treated with heparin may indeed be due to hepatocellular damage.

Heparin was associated with a concentration-dependent toxic effect (Fig. 1). Cells were treated with a wide range of heparin concentrations surrounding the apparent "therapeutic" concentrations (0.1 to 10 units/ml) required for anticoagulation [1]. The mild increase seen in LDH index is consistent with the clinical expression of this phenomenon. Patients treated with heparin who exhibit aminotransferase increases will generally show a 2- to 3-fold increase. In addition, no patients have shown coincident signs of acute hepatic dysfunction such as elevated bilirubin or jaundice [2–6]. There has been a report of a difference between pork and beef heparin preparations in their potential to cause aminotransferase elevations [5]. That study indicated that subjects exposed to pork heparin experienced an increased incidence of aminotransferase elevations compared to beef heparin. However, in these studies, no difference between beef lung and porcine intestinal mucosal heparin in causing elevation of the LDH index was detected. There was no induction of LDH enzyme synthesis as reflected by a lack of increase in total LDH in the system (intracellular plus extracellular LDH).

Exposure of the cells to [^{3}H]-heparin indicated that from 1.4 to 8.3% of the heparin in the culture medium is either taken up or bound to the cell. Previous investigations into the cellular localization of heparin following intravenous injection showed that the majority of heparin sequestered in the liver is present in sinusoidal cells with none observed in the liver parenchymal cells [20]. The methodology used in that study, however, may have precluded detection of heparin in the parenchymal cells. To

Table 2. Summary of the effects of albumin on the LDH index and heparin uptake in isolated hepatocytes*

Treatment	Change in LDH index (%)	Uptake of radioactivity (% of total)	
		Cytosol	Total cell
Control vs heparin (10 units/ml)	12.4 ± 4.3†	3.3 ± 1.3	5.3 ± 1.5
Control vs albumin (5 g/dl) + heparin	5.4 ± 3.4	2.8 ± 0.3	5.7 ± 2.1

* Values are expressed as mean ± S.D. (N = 5).

† Difference between control and heparin-treated plates ($P < 0.01$).

differentiate between heparin binding to the exterior surface of the cell and actual uptake, a number of experiments were conducted to isolate the plasma membranes. The distribution of [^{3}H]-heparin showed that minimal quantities were associated with the plasma membrane while the majority was located in the cytosolic fraction (containing cytosol, microsomes and mitochondria). This shows that small amounts of heparin are taken up by the hepatocyte, thereby increasing the possible mechanisms for the heparin-mediated toxicity.

Heparin binds to various plasma proteins including albumin [21]. Addition of albumin (5 g/dl) to the culture medium resulted in a decrease in the toxic effect of heparin at a concentration of 10 units/ml. Due to the small amount of intracellular heparin, we could not detect a difference in the amount of heparin in the cell following albumin treatment; however, the binding apparently reduced the availability of heparin to exert its toxic action. Perhaps this accounts for the apparent trivial injury seen in humans.

The mechanism of the demonstrated toxic effect of heparin remains unknown. Many compounds causing hepatocellular damage interact with the oxidative enzyme system resulting in metabolic activation to a toxic species [22]. Heparin metabolism is believed to occur primarily by a variety of sulfamidase and sulfatase enzymes present in the cells of the reticuloendothelial system where the majority of heparin sequestration occurs [1]. Sulfatases occur as two major types: an insoluble microsomal enzyme and a lysosomal enzyme [23]. These enzymes have been isolated from both rat and human liver, in addition to other tissues. Following desulfation of heparin, only the carbohydrate polymer remains. Therefore, the generation of a toxic species from heparin is doubtful. It has been demonstrated that the oxidative enzyme system deteriorates rapidly in cultured hepatocytes [10]. We used hepatocytes which were cultured for 24 hr prior to exposure to heparin. Therefore, the oxidative enzymes were probably not involved in the observed toxicity. Despite the lack of concentration of heparin on the plasma membrane, we cannot rule out a membrane-mediated effect.

Questions remain regarding the demonstrated toxic effect in cell culture and its relationship to the observed clinical effects of heparin. The majority of patients treated with heparin showed aminotransferase elevations which were characteristic of hepatocellular damage. The results in the cultured hepatocytes indicated that the effects seen *in vivo* may indeed reflect hepatocellular damage. It remains to be seen if the aminotransferase elevations seen in man represent clinically significant liver injury.

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