BASIC SCIENCES RESEARCH

Brevetoxin-3 (PbTx-3) on Mouse Liver Slices: a Histological Study

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ABSTRACT. Brevetoxin-3 (PbTx-3) is a marine toxin produced by the dinoflagellate *Ptychodiscus brevis*. Its effects on excitable tissues have been the main subject of studies , but little is known about how it affects non-excitable tissues. To study possible non-neural effects of PbTx-3 (78nM), its effects on hepatic cell structure *in vitro* were evaluated. PbTx-3 caused hypertrophy and increased vacuolation of hepatocytes, and an increase in basophilia in the perivenous area of the lobules. Ultrastructurally, it was evident that the vacuolation was related to swelling of the endoplasmic reticulum, changes that probably account for the increased basophilic reaction of the cells. The swelling in smooth endoplasmic reticulum, degranulation of rough endoplasmic reticulum, the deformities and lytic cristae in the mitochondria, and the presence of active lysosomes are evidence of the PbTx-3 effects upon liver cells. These responses are probably caused by the liver's detoxification role on the PbTx-3. *Keywords: Brevetoxin, liver slices, histological alterations.*

In many tropical and subtropical regions marine toxin poisoning ("fish poisoning") is a common cause of food poisoning in humans, that can cause illness and occasional death (1-5). Marine dinoflagellates neurotoxins are responsible for most of the reported food poisonings in these areas, thus extensive studies concerning the toxins produced by these organisms are important in delineating the mechanism by which they produce their deleterious and sometimes fatal toxicity. A group of these toxins are the brevetoxins (PbTx's), produced by the dinoflagellate *Ptychodiscus brevis*. They are lipid-soluble and their effect is excitatory, mediated by the enhancement of cellular Na^+ influx and binding on the voltage-sensitive Na^+ channel (6-8). They have been isolated, purified, and are now classified as PbTx-1 to 10 (7,8). PbTx-3 was used in this study because of its commercial availability and because most previous data and information on marine neurotoxins have been obtained primarily from studies of this PbTx-type.

Most studies on marine neurotoxins have been focused on their effects on excitable tissues (9-15). As they are highly lipid-soluble, and are absorbed through the gastrointestinal (GI) tract, they must be processed by the liver for elimination from the body, and the liver itself might become a target for the toxins or their metabolites. Studies on the pharmacokinetics of marine neurotoxins, including brevetoxins, have revealed that the liver is the major route of detoxification (16,17). In the case of brevetoxins, about 90% of an administered dose is excreted in six days as four polar compounds. As of today, little is known concerning the deleterious effects of these toxins on hepatic tissue. This is of utmost importance since the possible deleterious effects of brevetoxins on the liver could compromise their metabolism and subsequent excretion. Preliminary "in vitro" studies by our group (18,19) indicated that Brevetoxin-3 (PbTx-3) inhibits the oxygen consumption rate (QO_2) in liver slices, and increases the hepatocytes Na^+/K^+ ratio, raising the possibility that this toxin exerts direct effects on this tissue. These effects were abolished by the sodium channel blocker tetrodotoxin (TTx) (18,19), suggesting that in the liver, PbTx-3 can induce some effects that

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appear to be similar to those observed in excitable tissues. The information obtained indicates that during passage thru the liver PbTx-3affects physiological processes that might have a cytological impact with notable histological and ultrastructural consequences. This study was designed to determine the histological effects and/or ultrastructural alterations caused by PbTx-3 on the liver. To gain an insight of possible non-neural effects, mice liver slices were exposed acutely to the toxin and studied at the light microscope level. Evaluation of the toxin's effects upon several organelles usually affected by drugs and other toxins was done by electron microscopy.

Materials and Methods

Preparation of liver slices for morphological examination with the light and electron microscopes. Adult male CF_1 mice (Charles River Lab., Wilmington, MA), 8 to 10 weeks of age weighing 25-30 g were used. The animals were sacrificed by rapid cervical dislocation and the liver surgically removed. Liver slices of about 20-40 mg wet weight were prepared with a Staddie-Riggs microtome as described previously by Bernstein et al., (20). During preparation, the slices were kept at 37°C in 20 ml of an oxygenated Krebs-Ringer Bicarbonate buffer (KRB), pH 7.4, containing: 118 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$ and 10 mM Glucose. The slices were then preincubated for 15 min in 20 ml of the same buffer in a Dubnoff Metabolic incubator at 37 C and agitated at 80 strokes/min. The incubation medium was continuously bubbled with a O_2-CO_2 (95:5%) gas mixture to obtain a pH value of 7.4. Following the preincubation indicated above, liver slices used as controls were fixed prior to the addition of PbTx-3 (78nM), that is, at 0-time interval; experimental slices were fixed after 60 min exposure. Samples for light microscopy were fixed in 10% buffered neutral formalin, embedded in paraffin blocks, sectioned at 6 μm with a rotary microtome and stained by a routine hematoxylin and eosin method (21). For electron microscopy, the samples were fixed in a cold 2% paraformaldehyde-glutaraldehyde solution for 2 hr and then postfixed in 1% OsO_4 at 4°C for 1 hr. The samples were dehydrated in a series of graded concentrations of ethanol, embedded in Epon 812, and then sectioned with a diamond knife on a LKB III-model 8800 ultratome. The ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Phillips TEM-model 201 transmission electron microscope. Additional ultrathin sections to be used for general cell descriptions were stained with hematoxylin and eosin (H&E).

Liver cell size determinations. Liver cell size determinations were carried out by the method described by Tanaka et al., (22) with some modifications. The sections from controls and two different experimental animals were examined using a 10X ocular and a 40X objective. A 20x20 grid was placed in the ocular for a field area of $5mm^2$. In each section three fields were chosen at random. In each field, two cells were chosen to be measured from each even vertical row in horizontal direction for a total of 20 cells near the portal region and 20 near the central vein of the lobules. The base and height (b x h) were measured and the values used to determine the cell area in μm^2. Cell areas and the averages from each field were established and expressed as $\mu m^2 \pm$ SE.

Statistical Analysis. All results were expressed as means ± SE. The data obtained were analyzed using an analysis of variance. Differences between individual means were further analyzed by Scheff's F-test. The level of significance chosen was $p<0.05$.

Results

Regarding cell area, it was found that the average cell area was doubled in PbTx-3-treated slices (Table 1).

In the periportal region of the lobules, the cell area increased from 692.6 ± 38.3 μm^2 in controls to 1068.9 ± 26.4 μm^2 in PbTx-3 treated animals after 60 min of exposure to the toxin. In the perivenous zone the increase was from 604.5 ± 19.4 μm^2 in controls to 1181.3 ± 68.3 μm^2 in the treated slices. Both acidophilic and basophilic cells showed an increase in size; but in acidophils the increase was far more noticeable. The increases in cell areas were statistically significant. The cells of treated

Table 1. Effect of Brevetoxin-3 (PbTx-3) on the Hepatic Cell Area of Mouse Liver Slices

Conditions	Time	Hepatic zone	Cell area
	min		μm^2
Control	0	hepatic triad	690.80±24.4
	60		692.6±38.3
	0	central vein	603.4±20.1
	60		604.5±19.4
PbTx-3	0	hepatic triad	692.6±38.3
	60		1068.9±26.4*
	0	central vein	604.5±19.4
	60		1181.3±68.3*

The values shown are the means ± SE of 120 cell areas. PbTx-3 was used at a final concentration of 78nM. Statistical comparisons were performed between the control and brevetoxin-3-treated slices with $p<0.05$. differences ($p<0.05$).
*Significant differences ($p<0.05$) between the control and brevetoxin-3-treated slices.

slices showed not only an increase in size, but also increased vacuolation in the cytoplasm and strong basophilia in the perivenous region of the lobule. Vacuolation may have been caused by steatosis (fat retention), water, and/or protein retention (23,24). The control slices demonstrated an absence of the intense basophilia from the perivenous area (Figs. 1a, b). The increased basophilic reaction in PbTx-3 treated slices was mostly confined to the perivenous region of the hepatic

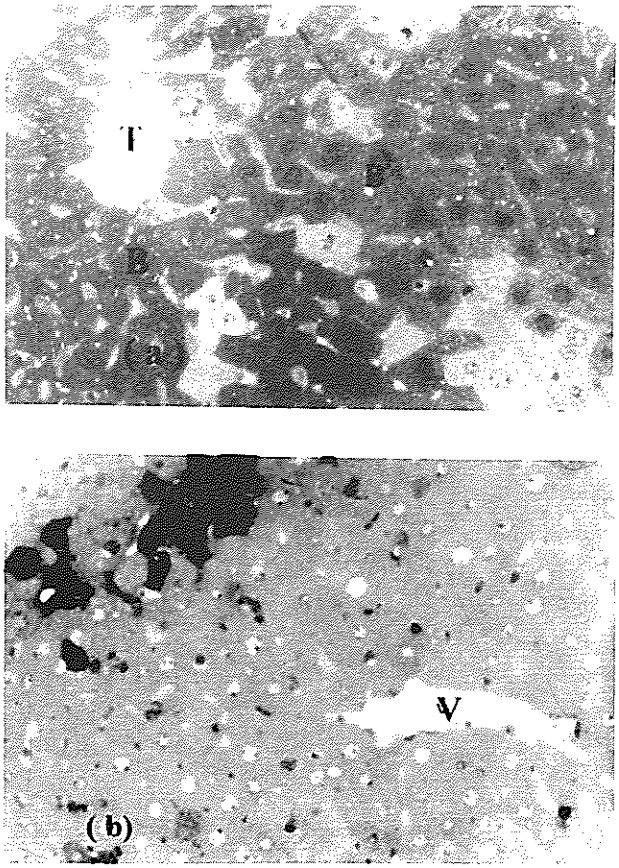


Figure 1. (a) Control liver-periportal area showing basophilic (B) hepatocytes. Hepatic portal triad (T). Epon 812, ultrathin. Hematoxylin and Eosin stain, 200X. (b) Control liver-perivenous area. Notice lack of basophilic cells around the central vein (V). Epon 812, ultrathin. Hematoxylin and Eosin stain, 200X.

lobule (Figs. 2a, b). This basophilia might be indicative of sites of increased amounts of rough endoplasmic reticulum (rER) and protein synthesis, as a normal hepatic metabolic response or a change in intracellular conditions brought about by changes in ion concentrations as a toxic effect. Vacuolation was present both in basophilic and eosinophilic cells (Figs. 3a, b).

Since drugs and toxins seem to affect lipid storage, the mitochondria, and the endoplasmic reticulum, studies of

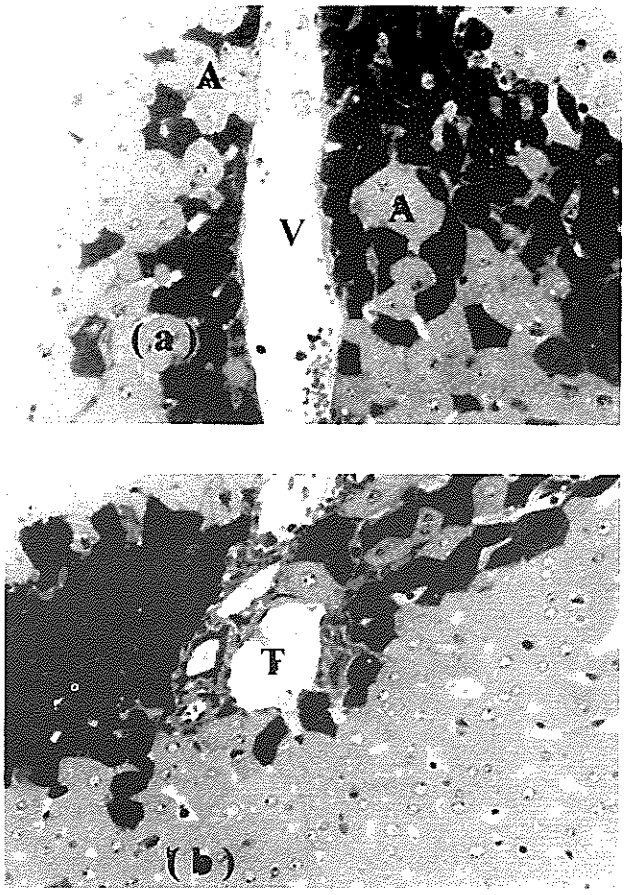
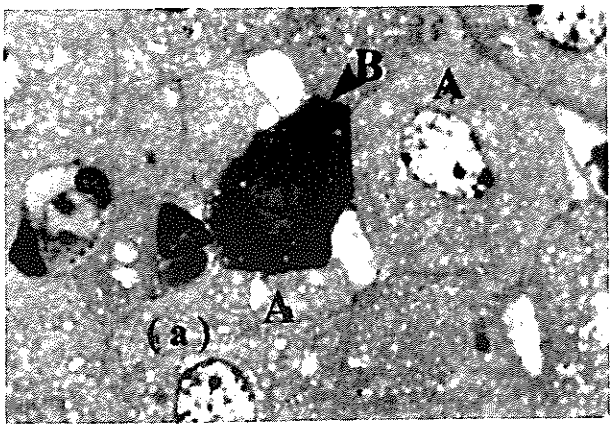
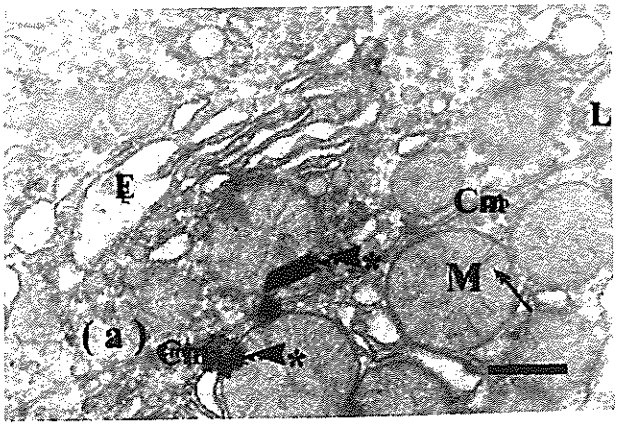


Figure 2. (a) PbTx-3 treated liver-perivenous area (60min). Acidophilic cells (A) show hypertrophy. Basophilic cells (B) surround the central vein (V). Epon 812, ultrathin. Hematoxylin and Eosin stain, 200X. (b) PbTx-3 treated liver-periportal area (60 min). Basophilic cells (B) are less numerous. Hepatic portal triad (T). Epon 812, ultrathin. Hematoxylin and Eosin stain, 200X.

hepatic ultrastructure were focused on these organelles in both control and PbTx-3-treated sections. In controls, acidophilic cells of the perivenous area showed the expected numerous mitochondria, both types of endoplasmic reticulum, being the rER more abundant (Fig. 4a). The mitochondrial cristae were of the lamellar type. Lipid droplets in the area were small but not numerous. In the periportal region both basophilic and acidophilic cells were present. Within the acidophilic cells (Fig. 4a), the mitochondria showed mostly lamellar cristae, the endoplasmic reticulum (ER) was rough, and sometimes in the form of microsomes. In the basophilic cells (Fig. 4b), the rER was far more abundant than in the acidophilic cells. Most mitochondrial cristae were lamellar. Some of the mitochondria showed signs of degeneration (Fig. 4b).Electron micrographs of the perivenous area of PbTx-3 treated slices show both basophilic and acidophilic cells present in the area. Within the basophilic cells (Fig. 5),





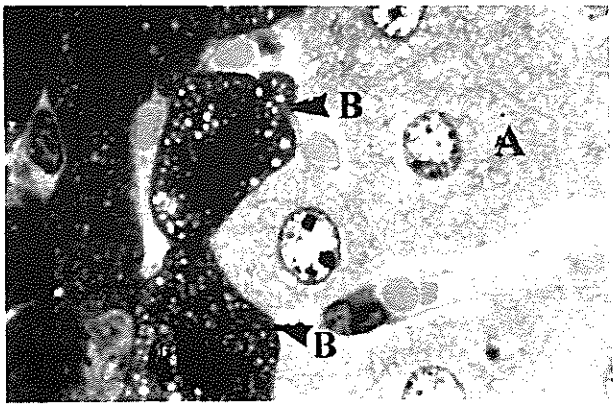


Figure 3. (a) Control hepatocytes-perivenous area I (60min). Acidophilichepatocytes (A). Basophilic hepatocytes (B). Notice lack of vacuolation in basophilic cells and vacuoles in acidophilic cells. Epon 812, ultrathin. Hematoxylin and Eosin stain, 1,000X. (b) PbTx-3 treated hepatocytes-perivenous area (60 min). Basophilic cells (B) are vacuolated, not clumped. Acidophilic cells (A) show few vacuoles in this area. Epon 812, ultrathin Hematoxylin and Eosin stain, 1,000X.

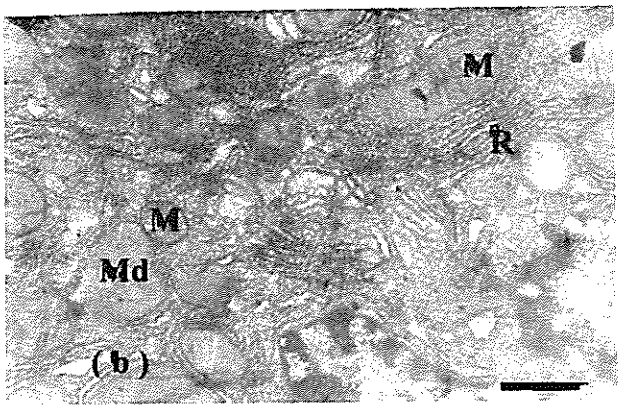


Figure 4. (a) Control hepatocytes-perivenous area II. General view. Cellmembrane (Cm). Mitochondrion (M). Cristae ( ). Endoplasmic reticulum (E). Lipid droplets (L). Contaminated area (*). 15,000X. (b) Control hepatocytes-basophilic cell. General view. Mitochondrion (M). Degenerated mitochondrion (Md). Notice abundance of rough endoplasmic reticulum (R). 15,000X. Bars: (a) 1μm. (b) 1μm.

few of the mitochondria were almost normal with lamellar cristae. Inside most of them paracrystalline bodies and lytic cristae were evident. Small lipid droplets were seen but their number was not increased. A degranulated form of rER was detected and few vesicles of sER were present, the endoplasmic reticulum was swollen. In normal sized acidophilic cells (Fig. 6), degranulation of the rER was accompanied by areas of accumulation of ribosomes. Profiles of rER were normal, but the highly vacuolated, hypertrophied acidophilic cell (Fig. 7) had deformed mitochondria of variable size and shape, with lytic cristae and some with tubular cristae. The rER was swollen; the sER was more abundant, and a few lipid droplets were larger than in controls. Lysosomes were also present in the hepatocyte cytoplasm, but no increase in their number or size, was evident as compared to controls. They are involved in intracellular digestion, an essential part of the hepatic defense and transport systems. Besides the lysosomes some autophagic vacuoles with myeloid bodies (Fig. 8) inside were seen in the cytoplasm of basophilic cells of PbTx-3 treated slides, and in less amount in the acidophilic cells.

Discussion

Drugs and toxins, while being processed by the liver for elimination, elicit a limited amount of responses that can be morphologically detected at the histological and ultrastructural levels. The initial and most common response reported is an increase in lipid storage or steatosis within the hepatocytes and the spaces of Disse leading to

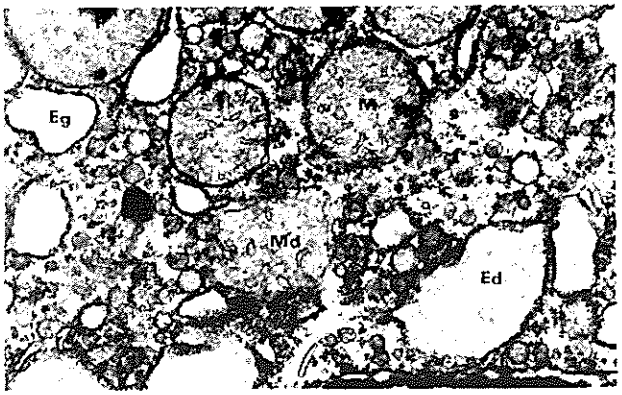


Figure 5. PbTx-3 treated hepatocytes-basophilic cell (60 min). General view. Mitochondrion with crystals (M). Degenerated mitochondrion (Md). Cristae ( ). Paracrystalline body (P). Granulated endoplasmic reticulum (Eg). Degranulated endoplasmic reticulum (Ed). Lipid droplets (L). 15,000X. Bar: 1μm.

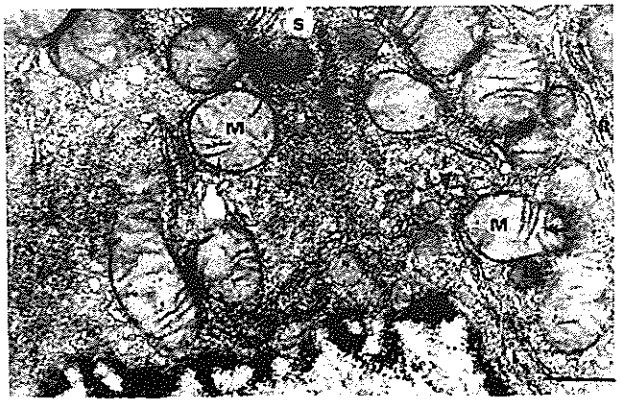


Figure 6. PbTx-3 treated hepatocytes-acidophilic cell I (60 min). General view. Mitochondrion (M). Cristae ( ). Smooth endoplasmic reticulum (S). Rough endoplasmic reticulum (R). Degranulated rough endoplasmic reticulum with ribosomes (*). Lipid droplets (L). Myeloid bodies or autophagic vacuoles (F). 15,000X. Bar: 1μm.

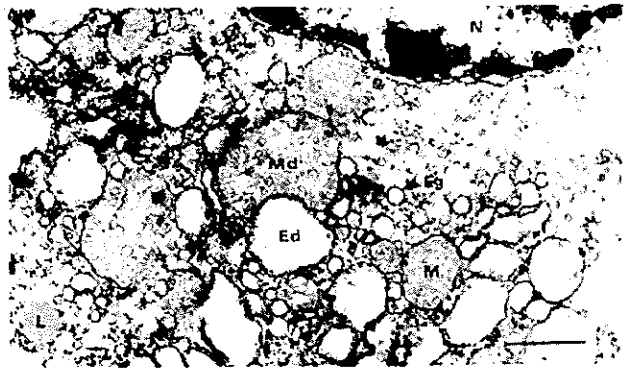


Figure 7. PbTx-3 treated hepatocytes-acidophilic cell II (60 min). General view. Nucleus (N). Mitochondrion (M). Degenerated mitochondrion (Md). Granulated endoplasmic reticulum (Eg). Degranulated endoplasmic reticulum (Ed). Lipid droplets (L). 15,000X. Bar: 1μm.

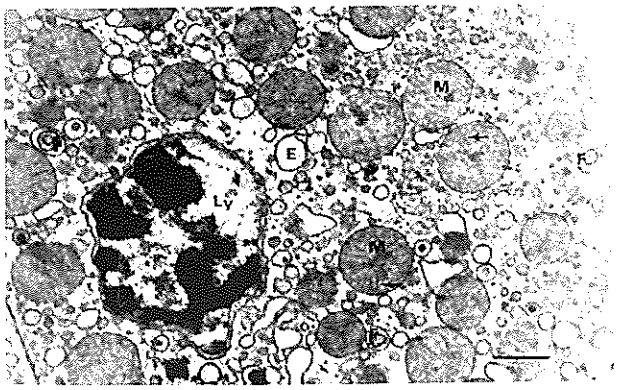


Figure 8. PbTx-3 treated hepatocytes-lysosome (60 min). General view. Lysosomes (Ly). Mitochondrion (M). Cristae ( ). Endoplasmic reticulum (E). Myeloid bodies or autophagic vacuoles (F). 10,000X. Bar: 1μm.

vacuolations and cell necrosis noticeable even at the level of light microscopy (25,26). The most affected organelles are the mitochondria, which show signs of degeneration, and the sER, which increases in amount (25). As such, it seems that PbTx-3 can be classified as an indirect hepatotoxic drug that affects both the metabolic and secretory functions of the hepatocytes. Within the liver, periportal areas are more aerobic than perivenous areas, and are deeply basophilic due to the numerous phosphate groups of the constituent ribosomal-RNA. These areas are also sites of active protein synthesis (26). The perivenous zones are associated with lipids synthesis. In rodents, contrary to humans, smooth endoplasmic reticulum (sER) is less abundant than rough endoplasmic reticulum (rER) in this area. Among the activities associated with the sER are lipid synthesis, re-esterification of free fatty acids into fat for storage, and the degradation of lipid-soluble drugs and toxic substances (26).

Ethanol and other drugs affect the sER, where drug metabolizing biochemical processes occur, and the usual response is an increase in quantity of sER. The slight increase of sER in the perivenous and periportal zones of PbTx-3 treated slices was no accompanied by a visible increase in lipid storage. As there was no evidence of steatosis around or within the liver cells or lobules, it is obvious that the toxin does not elicit a similar response as that caused by ethanol. The vacuolation seen at the light microscope level was not caused by increased fat storage in the form of lipid droplets, but by a swelling of the endoplasmic reticulum caused probably by retention of water and/or protein. However, within the cytoplasm autophagic vacuoles containing myeloid bodies were present. These bodies are also found after treatment with many drugs, and they mimic a lipid storage disease

associated with lysosomal alterations (25). The secondary lysosomes and the autophagic vacuoles present in the toxin-treated hepatocytes both provide for a mechanism of elimination of this exogenous material.

Mitochondria which are the chief energy source of the cell and the principal site of several enzyme systems were also affected. PbTx-3 had a lytic effect upon the cristae, specially those in the hypertrophied acidophilic cells. Their shape was altered and crystalloids were present in both cell types. The matrix granules, found anywhere in the intercristal spaces but often closely associated with the membrane of the cristae, are binding sites of calcium ions. They also increase the Ca^{++} intracellular concentration contributing directly to its internal regulation, and are also associated with lipid transport out of the liver (27). Crystal formation has been observed to start in these granules during pathological calcification in some cell types (28). Paracrystalline bodies were numerous in cells treated with PbTx-3. As they are involved in Ca^{++} or lipid regulation they may be found in normal mitochondria, and their increase in PbTx-3 treated slices must have occurred in response to an increase in intracellular Ca^{++} concentration. Perhaps they are involved in regulating lipid transport out of the toxin-treated cells, as there was no evidence of lipid accumulation.

An elevated intracellular Ca^{++} has been associated with an increase in intracellular proteins and other biomolecules, such as nucleic acids, that constitute pathological characteristics in many tissues (29-31). In other words, an increase in intracellular Ca^{++} concentration does lead to functional and structural alterations.

Hypertrophy of the cells, swollen cisternae of endoplasmic reticulum, mitochondrial structural changes with altered cristae, and prominent deposits of calcium, are other notable alterations that will be observed by an increase in intracellular Ca^{++} (31,32). As all these changes were observed in PbTx-3-treated hepatocytes, this observation supports the conclusion that PbTx-3 does indeed result in increases in intracellular Ca^{++} ion concentrations.

Drug-induced sER proliferation and the formation of vesicular cisternae; and rER degranulation and fragmentation are always a sign of toxic injury to the liver (25). Degranulation of the rER is least frequent in humans. The rER was more abundant than the sER in controls, and PbTx-3 did not induce a noticeable increase in sER. The rER in toxin-treated slices was observed to be swollen, causing hypertrophy of acidophilic cells mostly. Swelling on the endoplasmic reticulum and cisternae formation may be caused by water storage as a response to ion movements. Water could also account for the vacuolation seen at the level of the light microscope.

The basophilic reaction in the perivenous zone may be caused by changes within the cells related to intracellular ion concentration or protein accumulation. PbTx-3 by its mechanism of action increases the intracellular Na^+ concentration (6,7,8,18). This toxin probably activates another transport system that extrudes Na^+ from the cell in exchange for external Ca^{++}. In PbTx-3-treated liver cells the basophilia is related to degranulation of rER and the accumulation of ribosomes in the cytoplasm, a mechanism that increases the cellular protein content resulting in a basophilic reaction. PbTx-3 might inhibit other secondary transport systems, such as Na_o/H_i exchange; the inhibition of this system lowering the intracellular pH, resulting in a reduction of the metabolic activity of the cell. Proteins will be synthesized but not secreted, and therefore an accumulation of proteins will ensue. The increase in accumulated proteins could also account for the increase in size of the cell and the basophilia. The dilated rER will also be accountable for the observed increase in the size of cells.

In conclusion, under the light microscope the most noticeable effects were hypertrophy of the liver cells, an increase in vacuolation of the hepatocytes and in basophilia in the perivenous area of the lobules, all practically absent in control groups. Ultrastructurally, it was apparent that the vacuolation was related to swelling of the rER, with water and/or protein retention without accumulation of fat droplets. Accumulation of proteins and/or degranulated ribosomes account for the increased basophilic reaction of the cells, specially in the perivenous area, an area where lipids are normally processed. The action of PbTx-3 on liver slices, a non-excitable tissue, though resembling those found for excitable ones, requires more consideration for evaluation. It is possible that some active metabolites of this toxin are responsible for these effects, or that PbTx-3 may be acting directly on the liver cells. Additionally, it may require other elements of the hepatic structure, like associated blood vessels, nerves or the bile duct, for its action. If PbTx-3 elicits "in vitro" responses similar to those caused by acute or chronic exposure to other drugs and toxins, it is conceivable that these will also occur "in vivo". Therefore the "in vivo" effects of PbTx-3 on the liver need to be further investigated for comparative purposes.

Resumen

La brevetoxina-3 (PbTx-3) es una toxina marina producida por el dinoflagelado *Ptychodiscus brevis*. Sus efectos en tejidos excitables han sido su principal material

de estudio pero, se conoce poco sobre sus efectos sobre tejidos no excitables. Se llevaron a cabo estudios *in vitro* para de conocer los posibles efectos no neurales de la toxina PbTx-3 (78nM), sobre la estructura de células hepáticas. PbTx-3 causó hipertrofia y vacuolación de los hepatocitos; además de un aumento en basofília en las áreas perivenosas de los lobulillos. Ultraestructuralmente, fue evidente que la vacuolación está relacionada a un retículo endoplásmico dilatado, cambio que probablemente puede explicar el aumento en la reacción basofílica observada. La dilatación en el retículo endoplásmico liso, la degradación del retículo endoplásmico granuloso, la alteración y lísis en las cristas mitocondriales y la presencia de lisosomas activos son evidencia del efecto tóxico de PbTx-3 sobre las células del hígado. Estas respuestas probablemente son debidas al rol detoxificador del hígado sobre la PbTx-3.

Acknowledment

We would like to thank to Dr. Anarda González, Pathology Department, and the Central Electron Microscopy Unit at University of Puerto Rico Medical Sciences Campus for the help in providing their resources that made this project possible.

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