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Effect of a transitory ischaemia on the structure-linked latency of rat liver acid phosphatase and β-galactosidase

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The structure-linked latency of acid phosphatase and β-galactosidase was studied in rat liver lobes made ischaemic for 1 or 2h and then recirculated with blood for increasing periods. Free activity of acid phosphatase and unsedimentable activity of β-galactosidase are increased in homogenates of ischaemic livers. When ischaemia had been maintained for 1h, the recovery of normal latency for both enzymes was observed 1h after re-establishment of the blood flow. After a 2h period of ischaemia, unmasked activity markedly decreases during the first 1h after restoration of blood flow; after that, a large and irreversible secondary rise takes place. Chlorpromazine, injected 30min before or just after induction of ischaemia, extensively prevents the latency decrease occurring during restoration of blood flow. Modifications of the hydrolase distribution pattern obtained after differential centrifugation are in agreement with the latency changes. These results suggest that a 2h ischaemia causes an alteration of the liver lysosomes that is largely reversible and that restoration of blood flow induces an irreversible alteration of these organelles. Chlorpromazine treatment prevents the irreversible lesion from taking place.

When a rat liver lobe is made ischaemic during various time periods and afterwards circulation is restored, liver cell death may occur depending on the length of time for which the cell is deprived of blood. In general, when 2h ischaemia is followed by a re-establishment of the circulation, a large majority of the cells are histologically dead 24h later (Bassi & Bernelli-Zazzera, 1964; Chien & Farber, 1977). Treatment with chlorpromazine before inducing ischaemia prevents cell death (Chien *et al.*, 1977). Such an experimental system is appropriate for study of the metabolic disturbances which precede and accompany cell death and the mechanism by which a drug is able to oppose or to make reversible these modifications.

We have undertaken a study of the biochemical and morphological alterations of liver subcellular structures induced by a transient ischaemia. In the present paper we report that the structure-linked latency of two lysosomal hydrolases, acid phosphatase and β-galactosidase, is greatly affected by this process and that chlorpromazine treatment is to some extent able to prevent latency modifications.

Experimental

Transitory ischaemia was obtained by the method of Chien & Farber (1977). Male Wistar rats weighing 200–300g were laparotomized under diethyl ether anaesthesia, and the vascular pedicle of the left lobe was clamped with small forceps. To re-establish the blood flow, the animals were again anaesthetized, the abdomen was reopened, the forceps removed and the wound closed. The animals were killed at various times after restoration of blood flow, the left liver lobe was removed, weighed and homogenized in ice-cold 0.25M-sucrose, by three strokes of a motor-driven Teflon pestle in a Potter homogenizer (A. H. Thomas, Philadelphia, PA, U.S.A.). The homogenized suspension was adjusted to a volume corresponding to ten times the weight of the tissue. Part of the homogenate was centrifuged for 40min at 40000rev./min, in the no. 40 rotor of the Spinco preparative ultracentrifuge.

Free and total acid phosphatase (EC 3.1.3.2) activity was measured in homogenates as described by Wattiaux & de Duve (1956); total β-galactosidase (EC 3.2.1.23) activity was determined in homogenates and supernatants by the method of Vaes (1966). Protein was determined by the method of Lowry *et al.* (1951). A unit of enzyme activity is defined as the amount of enzyme causing the decomposition of 1μmol of substrate/min under the conditions of the assay.

Homogenates were fractionated by differential centrifugation as described by de Duve *et al.* (1955),

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giving a nuclear fraction N, a heavy-mitochondrial fraction M, a light-mitochondrial fraction L, a microsomal fraction P and a soluble fraction S. Total acid phosphatase, β-galactosidase activities and protein were measured in each fraction.

Blood was withdrawn from the posterior vena cava with a syringe rinsed with 0.9% NaCl containing 1 mM-EDTA, pH 7.4.

Results

The integrity of lysosomes in homogenates was assessed by measuring the free activity of acid phosphatase and the unsedimentable activity of β-galactosidase. Free rather than unsedimentable acid phosphatase was selected because, after being released in a particulate fraction, this enzyme is easily re-adsorbed on subcellular structures (de Duve & Beaufay, 1959; Baccino *et al.*, 1971). Thus unsedimentable acid phosphatase is an underestimate of the unmasked amount of this enzyme. On the contrary, most of the β-galactosidase released from the lysosomes in a homogenate is not re-adsorbed and is recovered in the unsedimentable fraction (Baccino *et al.*, 1971).

Control experiments

In several control experiments, we found that free acid phosphatase, unsedimentable β-galactosidase and the total activities of both hydrolases were not significantly modified in the homogenates of unligated liver lobes of operated animals, independent of the time of blood-flow restoration and whether or not the rats were injected with chlorpromazine. These activities do not differ from those found in the livers of unoperated animals. For the above reasons, the values found for the livers of unoperated animals are only given as controls in the following sections.

Effect of 1 h ischaemia

As illustrated by Fig. 1, the free activity of acid phosphatase and the unsedimentable activity of β-galactosidase increase in the homogenates of liver lobes subjected to 1 h ischaemia. The process is largely reversible by reperfusion; no more than 1 h after restoration of blood flow, almost normal free and unsedimentable activities are recovered.

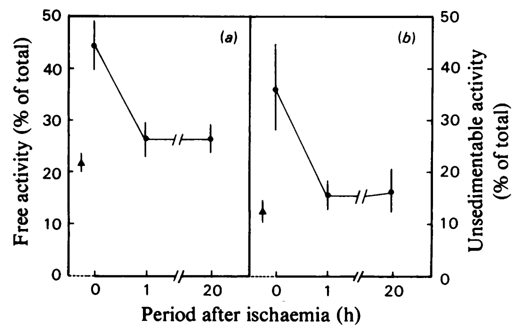

Fig. 1. *Influence of 1 h ischaemia on the free activity of acid phosphatase (a) and the unsedimentable activity of β-galactosidase (b)*

These activities are given as percentages of the total activity. The abscissa shows time (h) after re-establishment of the blood flow. ▲, Value corresponding to average free or unsedimentable activities found in homogenates of normal lobes. Means ± S.D. for at least five animals are presented.

Effect of 2 h ischaemia

Free activity of acid phosphatase and unsedimentable activity of β-galactosidase are markedly increased after a 2 h ischaemia (Fig. 2). During the first 1 h after the re-establishment of the liver blood flow, both enzymes tend to recover normal latency without, however, reaching the values found in non-ischaemic liver homogenates. Later, free acid phosphatase and unsedimentable β-galactosidase activities undergo a large secondary rise. Data obtained when rats had been injected intraperitoneally with chlorpromazine before the liver lobes were made ischaemic, are also shown in Fig. 2. The increase in free and unsedimentable activities is

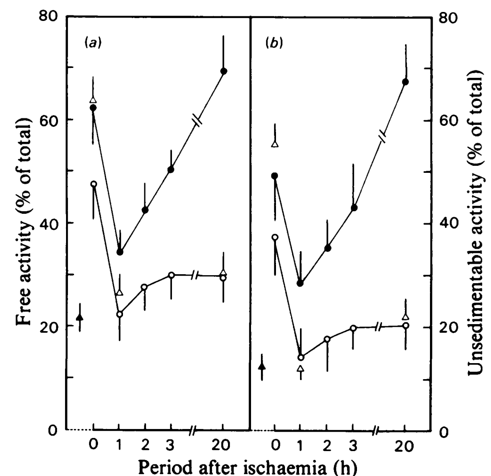

Fig. 2. *Influence of 2 h ischaemia on the free activity of acid phosphatase (a) and the unsedimentable activity of β-galactosidase (b)*

These activities are given as percentages of the total activity. The abscissa shows time (h) after re-establishment of the blood flow. ▲, Value corresponding to average free or unsedimentable activities found in homogenates of normal lobes. ●, Untreated rats; ○, rats injected with chlorpromazine (2 mg/100 g body wt.) intraperitoneally, 30 min before induction of ischaemia; △, rats injected with chlorpromazine (2 mg/100 g body wt.) subcutaneously, just after induction of ischaemia. Means ± S.D. for at least five animals are presented.

less pronounced for the treated rats. The difference between the two groups of animals is particularly striking after re-establishment of the blood flow. Chlorpromazine treatment causes a recovery of normal latency for both hydrolases 1h after restoration of blood flow. After that, a slight secondary rise takes place, but it is not comparable with the rise observed in the untreated rats. Chlorpromazine injected subcutaneously just after ischaemia induction does not affect the increase in free and unsedimentable activities observed before reflow (Fig. 2). On the other hand, the prevention of the secondary rise of these activities after blood reflow is clearly apparent.

Total activity

The total activity of enzymes, expressed in units/g of protein, does not change when liver lobes are made ischaemic during 1h. After a transient ischaemia of 2h, a significant decrease in the total acid phosphatase activity is observed in the lobes re-perfused for 20h (Table 1); β-galactosidase total activity does not change. Treatment with chlorpromazine prevents the decrease in acid phosphatase total activity.

Distribution after differential centrifugation

The biphasic change in hydrolase latency as well as the chlorpromazine effect are also clearly illustrated by the enzyme distributions observed after differential centrifugations (de Duve *et al.*, 1955), exemplified by the distribution pattern of β-galactosidase (Fig. 3). In normal liver lobes, β-galactosidase is mainly recovered in the M and L mitochondrial fractions, with a peak of relative specific activity in the light-mitochondrial L fraction. Just after 2h ischaemia, the proportion of enzyme recovered in the mitochondrial fractions is markedly lower, whereas that found in the soluble S fraction is increased. At 1h after restoration of blood flow, recovery of a more normal distribution is obvious. Later, a progressive loss of β-galactosidase from the mitochondrial fractions takes place, with a parallel increase in the S fraction. When the rat had been treated with chlorpromazine, the distribution changes observed after 2h ischaemia without restoration of blood flow are similar to those found in

Table 1. *Total activity of liver acid phosphatase and β-galactosidase*

Activities are expressed in units/g of protein, as means ± S.D. ($n = 7$). A, normal liver; B, liver lobe was made ischaemic for 2h then blood flow was restored for 20h; C, liver lobe was made ischaemic for 2h, then blood flow was restored for 20h, but animals were injected with chlorpromazine (2mg/100g body wt.) 30min before induction of ischaemia.

Enzymes	A	B	C
Acid phosphatase	35.9 ± 5.1	26.3 ± 4.9	39.0 ± 11.0
β-Galactosidase	3.1 ± 0.6	3.2 ± 0.7	3.7 ± 1.0

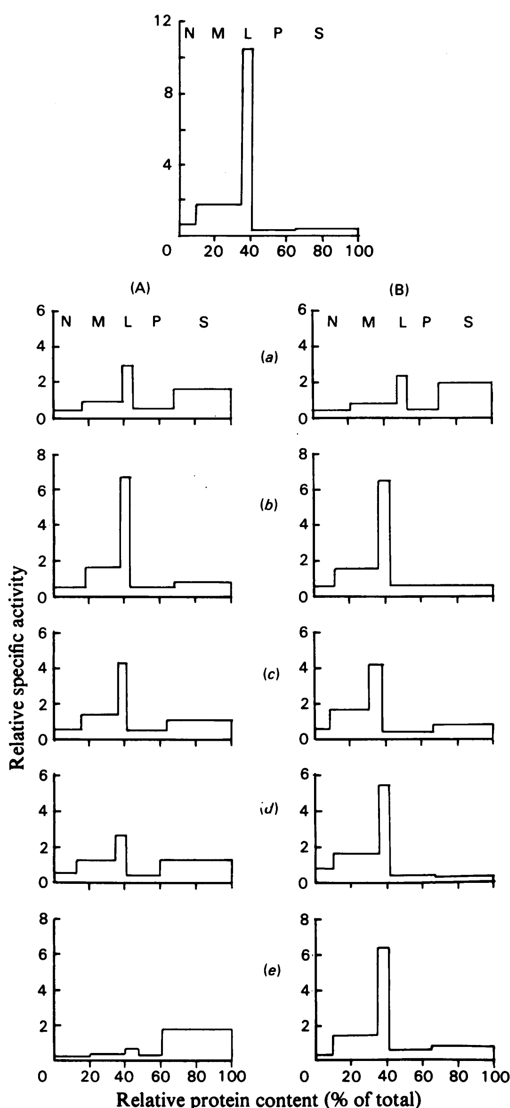

Fig. 3. *Distribution pattern of β-galactosidase after differential centrifugation by the procedure of de Duve et al. (1955)*

At the top is shown the distribution observed for an unligated lobe. The other distributions were obtained with liver lobes made ischaemic for 2h and then re-perfused during (*a*) 0h, (*b*) 1h, (*c*) 3h, (*d*) 6h, (*e*) 22h. (A) Untreated rats; (B) rats injected with chlorpromazine just after pedicle ligature. Ordinate, relative specific activity (percentage of total recovered activity/percentage of total recovered proteins); abscissa, relative protein content of fractions (cumulatively from the left to the right). Recovery values ranged from 94 to 102%.

the untreated rats. On the other hand, the normal distribution recovered 1h after re-establishment of the blood flow persists: the hydrolase remains chiefly in the mitochondrial fractions. Similar results (not shown) are obtained for acid phosphatase.

Blood plasma concentrations of enzymes

Blood plasma concentrations of acid phosphatase and β-galactosidase were measured at different times after re-establishment of the blood flow in liver lobes made ischaemic during 2h. The results are presented in Fig. 4. In animals untreated with chlorpromazine, the enzyme plasma concentration increases rapidly just after restoration of blood flow and is about doubled after 20min. After this, a slow increase continues to take place and a plateau is reached 1h after recirculation. Then the plasma concentration of both hydrolases remains almost constant for several hours. At 20h after restoration of blood flow, β-galactosidase is almost normal, whereas the acid phosphatase concentration remains higher than in normal blood. The modifications of the plasma hydrolase concentration after re-circulation are less pronounced when the rats have been injected with chlorpromazine. It is important to note that the concentration of both hydrolases in plasma is markedly lower than the concentrations existing in liver tissue, even when they are increased after recirculation. The maximum concentration that we found is 0.05 unit/ml of plasma for acid phosphatase and 0.0035 unit/ml of plasma for β-galactosidase, which is about 200 times lower than what is found per g of liver.

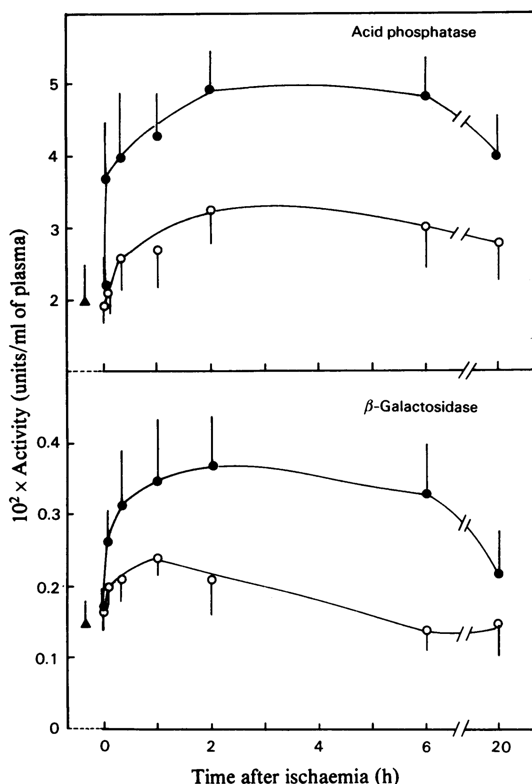

Fig. 4. *Plasma concentrations of β-galactosidase and acid phosphatase at different times after re-establishment of the blood flow in liver lobes made ischaemic during 2h*

Blood was withdrawn from the posterior vena cava with a syringe rinsed with 0.9% NaCl containing 1mM-EDTA, pH 7.4. Units of enzymic activity are defined as the amount of enzyme causing the decomposition of 1 μmol of substrate/min under the condition of the assay. The abscissa shows time (h) after re-establishment of the blood flow. ▲, Value corresponding to average activity found in normal blood plasma. ●, Untreated rats; ○, rats injected with chlorpromazine (2mg/100g body wt.) subcutaneously just after induction of ischaemia. Means ±S.D. for at least four animals are presented.

Discussion

An increase in the free or unsedimentable activity of the lysosomal enzymes results from a change in the lysosomes allowing free access of the enzymes to substrates present in the medium. An increase in the unsedimentable activity particularly supposes that a true disruption of the lysosomal membrane occurs, thus causing the release of the hydrolases to the external medium. Such a release may take place *in vivo* or could originate from an increased fragility of the granules, making them more easily disrupted by the homogenization procedures.

The latency modification of acid phosphatase and β-galactosidase resulting from 1h ischaemia quickly disappears after restoration of blood flow, indicating that lysosomal lesions induced in these conditions are reversible. The alteration of acid phosphatase free activity and β-galactosidase unsedimentable activity in liver lobes subjected to 2h ischaemia is biphasic. The suppression of the circulation causes a striking increase in these activities; the restoration of blood flow first allows the recovery of a relatively low free and unsedimentable activity, afterwards the amount of bound enzyme decreases. Apparently a 2h ischaemia induces a lysosomal lesion largely reversible at the start of restoration of blood flow, then the lysosomes are subjected to an irreversible alteration. The chlorpromazine effect suggests that the irreversible lesion does not depend on the reversible one. Indeed, when the drug is injected after pedicle ligature, no effect is observed on the first peak of unmasked activity, when the secondary rise is largely prevented.

The initial decrease in hydrolase latency probably

does not originate from release of these enzymes within the cell. Indeed, it is difficult to imagine by what mechanism hydrolases, free in the cytosol, could be reassociated with lysosomes no more than 1h after blood reflow. One possibility is that hydrolases are released outside the cells during the ischaemic period and recaptured when the blood flow is re-established. Obviously, if hydrolases are released outside the cells in ischaemic lobes, they will be recovered in a free form in the homogenates. If such a situation had occurred, an important and transient increase of blood plasma β-galactosidase and acid phosphatase could be expected just after restoration of blood flow, owing to the draining of these enzymes into the general circulation. In fact, an increase of both hydrolases takes place in blood plasma soon after re-establishment of the blood flow, but it is too small to be related to the amount of these enzymes present in free form in the ischaemic lobes. As these lobes weigh about 3g, one may calculate that about 17 units of acid phosphatase and 0.9 unit of β-galactosidase are free in the homogenate of a whole lobe. If this activity had been present in the ischaemic-lobe blood and was carried away in the general circulation after restoration of blood flow, it would have increased the plasma β-galactosidase to 0.09 unit/ml and the plasma acid phosphatase to 1.7 units/ml, assuming a plasma volume of 9.8ml (6% of the rat weight). The maximum concentrations of β-galactosidase and acid phosphatase that we found in the plasma are 0.0035 and 0.05 units/ml respectively. Moreover, the persistence of a relatively constant concentration of both hydrolases in the plasma for at least 2h after readmission of the circulation does not agree with the hypothesis of a significant recapture of these enzymes by the liver during the first hours after restoration of blood flow. It seems therefore highly improbable that acid phosphatase and β-galactosidase recovered free in ischaemic-lobe homogenates correspond to enzyme released in the lobe blood during ischaemia. A more plausible explanation for the release of acid hydrolases observed just after ischaemia is that it results from a lysosomal membrane disruption in the course of homogenization owing to increasing fragility of these organelles. A possible contributory factor to increased fragility is an increase in the size of the lysosomes, rendering them more easily disrupted by the shearing forces developed by the homogenizer. Such enlargement of the lysosomes has been observed in different pathological situations (Baccino, 1978). It is easily recognized by the cytochemical detection of acid phosphatase. Preliminary cytochemical observations indicate that lysosomes are not enlarged after 2h ischaemia. Thus, if the release of acid phosphatase and β-galactosidase results from increased fragility of the lysosomes, this fragility is probably not caused by the swelling of these organelles.

The hypotheses that we have discussed may also be considered to explain the secondary rise of acid hydrolase free activity after the restoration of blood flow. But in this case the possibility of a true release within the cell cannot be excluded, owing to the irreversibility of the phenomenon. After several hours of recirculation, a large proportion of hepatocytes die; release of the lysosomal contents into the cytosol could be a determining factor of cell death. It should be noted that during this period the total activity of acid phosphatase significantly decreases without modification of the total activity of β-galactosidase. This is related to the observations by de Duve & Beaufay (1959) on liver lobes subjected to definite ischaemia. These authors have shown that cell death is accompanied by the inactivation of various enzymes, including acid hydrolases, but that the extent of this inactivation as a function of the time of ischaemia may vary markedly from one enzyme to another. No explanation has been found for this, but it is possible that when the cell undergoes necrosis, the enzymes are subjected to a denaturation owing to the alteration of the cellular medium, resulting in a loss of their catalytic activity. Such a denaturation may be more rapid for certain enzymes, owing to their structure, their requirement for a prosthetic group etc.

The main effect of chlorpromazine is to protect the lysosomes from irreversible alteration after restoration of blood flow. We do not know if chlorpromazine acts directly or indirectly on lysosomes. Chien *et al.* (1978) proposed that the fundamental lesion induced by ischaemia is an alteration, at first reversible, of the permeability of the plasma membrane. This could be responsible for an influx of Ca^{2+} into the cell, leading to an activation of endogenous phospholipases and a degradation of the membrane phospholipids. As a result, the permeability of the plasma membrane would rise markedly on reperfusion, and a massive entry of Ca^{2+} would occur into the cell, inducing irreversible metabolic and structural alterations. Chlorpromazine could inhibit the phospholipases (Kunze *et al.*, 1976). Such a mechanism could be involved in the alterations of lysosomes during restoration of blood flow and the effects of chlorpromazine on this process. Ca^{2+}-activated endogenous phospholipases can bring about a disruption of the lysosomal membrane and a release of hydrolases within the cell. Such a phenomenon can be a major contribution to the irreversible events leading to cell death, because of the action of lysosomal hydrolases on cellular components. By preventing the phospholipase activation, chlorpromazine would oppose lysosomal membrane alterations and cell death.

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