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MODIFICATIONS IN ENTEROCYTE DIAMINE OXIDASE DISTRIBUTION INDUCED BY HEPARIN IN THE RAT

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Abstract—Heparin releases diamine oxidase (DAO) of enterocytic origin from binding sites located on small bowel microvascular endothelium.

In the villus tip enterocytes the enzyme is found in organelles (about 60%) and in cytosol (about 40%), while a negligible activity is present in the brush border. In this study we assessed the changes in DAO distribution into the enterocytes induced by a high dose of intraperitoneal heparin (1000 IU) in the rat, by assaying DAO activity on subcellular fractions obtained from ileal mucosa homogenate.

Heparin injection induced a marked reduction of enzyme activity in the S2 fraction (cytosol): after 30 min less than 20% of DAO activity is still found and only 8% after 150 min. In the P1 fraction (organelles) DAO activity significantly decreased only after 60 min and a further consistent reduction was recorded after 150 min.

Recovery of DAO activity was complete 4 days after the injection, though it was already clearly evident in the first 2 days. These results indicate that enterocytic DAO is distributed in two different compartments: DAO located in the cytosol is quickly released by heparin, while the organelles-linked enzyme is more slowly released. The finding that recovery in DAO activity happens earlier in the P1 fraction suggests that the enzyme supplies the cytosol after being synthesized in the enterocyte organelles.

The small intestinal mucosa is the main body store of the enzyme diamine oxidase (EC 1.4.3.6), both in man and in many animal species [1]. All other tissues in the rat, except for the placenta in the pregnant animal, contain less than 5% of the DAO activity of the intestinal mucosa [2]. This enzyme is mainly synthesized in the enterocytes of the villus tip rather than in proliferating cells of the crypt [3] and is then transported to binding sites [4], which are thought to be located on the intestinal vasculature [5]. In a previous publication [6], we have shown that the subcellular localization of the enzyme in the enterocytes is almost exclusively in the organelles (about 60%) and cytosol (about 40%) of the enterocytes and is barely detectable in the brush border.

It also has been shown that heparin, administered by different routes (intravenously, intraperitoneally, subcutaneously, or intrapulmonary), induces a rise in plasma DAO activity in man [7–9], and in many animal species [10–13], by releasing the enzyme from its binding sites [4], rather than directly from parenchymal cells [5]. In man [9, 14] and the rat [11], plasma postheparin DAO activity is a sensitive and quantitative marker of small bowel mucosa damage.

The aim of this study was to assess changes in the sub-cellular distribution of DAO induced by heparin injections in the rat; results could allow a better understanding of mechanisms involved in the enzyme release promoted by heparin and define the presence of different cell compartments of intestinal DAO.

MATERIALS AND METHODS

Experimental design. Thirty-seven male Wistar rats, weighing 280–300 g, were used. With the exception of the control group (seven rats), they were divided into groups of five; all rats were given regular laboratory rat chow and water *ad libutum* throughout the experiment.

With the exception of the control group, all the other groups of rats were injected intraperitoneally with 1 ml of saline containing 1000 IU of heparin. The control group received only saline.

The animals were anaesthetized with diethyl ether and blood was withdrawn by cardiac puncture; then rats were killed by decapitation 30, 60, 150 min, 1, 2 and 4 days after the injection. Twenty-five centimetres of ileum (the segment with the highest DAO activity) proximal to the ileocecal value was removed, gently washed with cold saline and opened longitudinally. Subcellular fractions were obtained by the method of Schmitz *et al.* [15]. The mucosa was carefully scraped, homogenized and diluted 1/100 in 50 mmol/L mannitol and 2 mmol/L Tris buffer, pH 7.1; solid $CaCl_2$ added to a final concentration of 10 mmol/L. A 10 min centrifugation at 2000 *g* yielded a precipitate (P1 fraction) containing organelles and other cell membranes. The supernatant (S1 fraction) was centrifuged at 20,000 *g* for 20 min, obtaining a pellet (P2 fraction) with the brush border fragments and a final supernatant (S2 fraction) containing the cytosol.

The P2 fraction was not assayed for DAO because of the negligible amount of DAO activity present in the brush border membranes (about 2% of the total homogenate) [6]. The sum of DAO activities in the P1 and S2 fraction was never less than 90% of that present in the homogenate.

DAO assay. DAO activity was assayed by the method of Okuyama and Kobayashi [16] with slight modifications on plasma, total mucosa homogenate and P1 and S2 fractions.

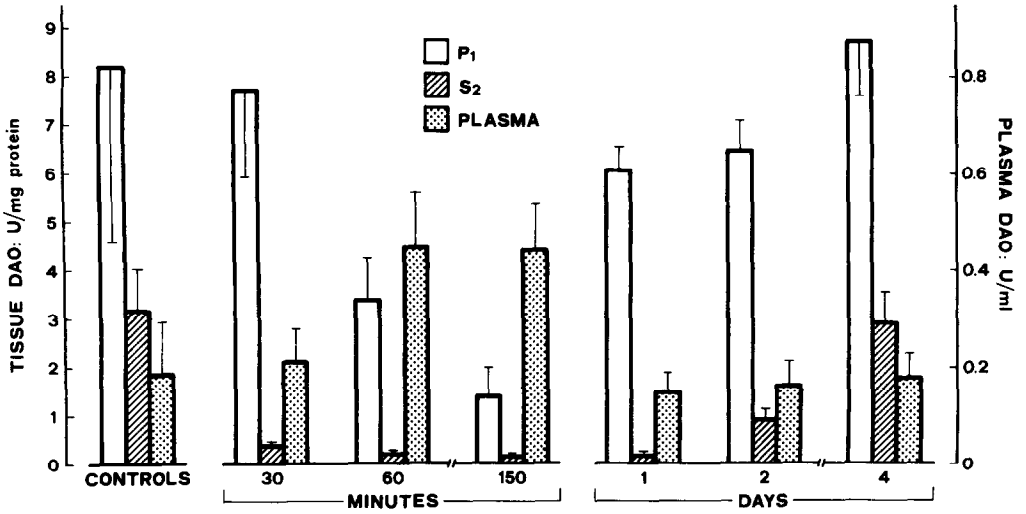

Fig. 1. Changes in DAO activity assayed in plasma and subcellular fractions of small bowel mucosa at different times after an intraperitoneal heparin injection (1000 IU) in rats. Bars represent standard deviation.

In a final volume of 2.1 ml, the assay mixture contained: 1.5 ml sodium phosphate buffer, 0.1 mol/L, pH 7.2; 0.3 ml sample; 0.2 ml, 0.4 mol/L acetaldehyde [17] and 0.1 ml substrate, a mixture of putrescine dihydrochloride and 1,4-^{14}C putrescine dihydrochloride up to a concentration of 5 nmol per tube with an activity of 0.1 μCi.

The samples were incubated for 60 min at 37°. Labeled reaction product, ^{14}C-Δ1-pyrroline, was directly extracted in a toluene-based scintillation mixture (1 L of toluene with 0.1 g of 1,4-bis-2(4-methyl-5-phenyloxazoyl)benzene and 6 g of 2,5-diphenyloxazole) and assayed for ^{14}C by a Packard Tricarb 4530 scintillation counter.

Assay blanks were prepared by incubation of samples in the presence of 2×10^5 mol/L aminoguanidine, a specific inhibitor of DAO activity [16].

The labeled putrescine hydrochloride was purchased from Radiochemical Centre (Amersham, U.K.); unlabeled putrescine dihydrochloride, acetaldehyde and aminoguanidine were obtained from Fluka AG (Buchs, Switzerland).

DAO activity in plasma was expressed as U/ml plasma and in tissue homogenates as U/mg protein (1 U = 1 nM of putrescine dihydrochloride oxidated in 1 hr at 37°, pH 7.2). Protein content was determined on the total homogenate and on the subcellular fractions by Lowry method [18].

Statistical analysis. Rank-sum test for unpaired data was used to analyze the difference in enzyme activities at the different times of the experiment.

Results are expressed as mean ± standard deviation.

RESULTS

Figure 1 shows the changes in plasma and mucosal DAO activity at different times after the heparin injection.

Decline in enzyme activity

There was a prompt fall in enzyme activity in the S2 fraction; the reduction at 30 min ($P < 0.005$ when compared with control values) was followed by a further slight drop at 60 ($P < 0.005$) and 150 min ($P < 0.005$). Thirty minutes after the heparin injection there was less than 20% of the baseline DAO activity and after 150 min only 8% in the S2 fraction.

In the P1 fraction DAO activity decreased significantly ($P < 0.005$) only after 60 min with a further consistent reduction after 150 min ($P < 0.005$). In fact, although more than 90% of the enzyme activity was still present in this fraction at 30 min, by 60 and 150 min the percentage had dropped to about 40 and 20% respectively.

High plasma DAO values were observed 60 and 150 min after the intraperitoneal heparin injection (0.45 ± 0.11 and 0.44 ± 0.09 U/ml respectively). A slight increase was already evident at 30 min (0.21 ± 0.07; basal values 0.18 ± 0.1).

Recovery of enzyme activity

One day after beginning the experiment, a relevant of enzyme activity was seen in P1 fraction ($P < 0.01$ when compared with the lowest values recorded at 150 min) while no statistically significant change in DAO activity was recorded in S2 fraction. Two days after the injection a partial recovery of enzyme activity was seen in both fractions, though it was more marked in the P1 fraction. Complete recovery was observed at day 4.

Twenty-four hours after heparin injection plasma DAO values were similar to those seen in the controls and no significant modifications were observed in the following days.

DISCUSSION

In mammals, DAO is located almost exclusively in villous tip enterocytes [3] and its activity is not confined to the brush border [6]. Plasma DAO activity, normally very low, increases after administration of heparin which displaces the enzyme from

binding sites, located on intestinal microvascular endothelial cells [4], probably continuously supplied with the enzyme stored into the enterocytes. In this study, we have investigated the sequence of changes in intracellular DAO distribution after a very high dose of heparin chosen in accordance with Luk *et al.* [11]. These authors found that, in the rat, DAO release prompted by heparin is dose dependent; in fact, there was a linear relationship between the maximal post heparin plasma DAO activity and the heparin dose used (ranging from 4 to 4000 U/kg of body weight).

Our results showed that 30 min after an intraperitoneal injection of heparin very little DAO remained in the cytosol (S2), while the DAO found in the organelles (P1) was only slightly reduced. Later, a further reduction in DAO activity was seen in the P1 fraction: after 150 min, the remaining enzyme activity accounted only for 20% of the P1 fraction and for 8% of the S2 fraction.

Complete recovery in DAO activity occurred 4 days after the heparin injected but a definite increase was already observed in P1 fraction at day one and also in the S2 fraction after 2 days.

Our data are consistent with the hypothesis that the enzyme in the cytosol of the enterocyte rapidly and continuously supplies DAO binding sites on microvascular endothelium which, in turn, are depleted by heparin. This hypothesis is also supported by the finding of high plasma DAO values early after intravenous heparin injection in humans [8, 9]. Conversely, DAO present in the P1 fraction is released only when practically all the enzyme in the cytosol has disappeared. Moreover, the finding that DAO recovery happens earlier in the P1 fraction suggests that the enzyme supplies the cytosol after being synthesized in the enterocyte organelles.

In summary, our study demonstrates the presence of two enterocyte compartments of DAO, each showing a different pattern of release following heparin injection: the enzyme in the cytosol compartment is released rapidly, while that linked to the organelles is released more slowly. Further studies are needed to clarify whether the complete recovery in DAO activity observed, 4 days after a massive cell depletion of the enzyme, results from a complete renewal of ileal epithelial cells (which takes about 3 days in the ileum [19]), or from *de novo* synthesis by the depleted enterocytes.

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