Intestinal Occlusion: Which Are The Modification Of Enzymatic And Ionic Activity? A Pathophysiologic Study.

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Abstract

In this work modifications occurring in the enterocytes, after intestinal occlusion of 120 minutes, have been studied.
We paid attention to alterations of the enzymatic activities of the following enzymes: maltase, leucin-aminopeptdase, alkaline phosphatase (markers of the brush border membrane) and Na+-K+-ATPase(marker of the brush border membrane); then we paid attention to alterations of K+ ion permeability of the brush border membrane.
The results show a marked alteration of all these enzymes under occlusion and the impossibility, after an occlusion of 120 minutes, for a functional recovery of the cells damaged by the intestinal occlusion.

The reported data are been extrapolated from the Thesis of R. Lovero

INTRODUCTION

Occlusive pathology is one of the most important pathologies of small intestine; in fact in the clinical practice, often urgently, a lot of patients are affected by mechanic ileum of different origin.

Whatever the cause may be, pathologic situation is always the same: a tract of the intestine is overstretched, seriously damaged and with no defined limits bordering surely vital areas.

During an intestinal occlusion the most remarkable alteration is the reduction of mucosal capillarity which is greatly reduced by distension of the intestinal wall and by opening of submucosal shunts.

We have investigate the alterations induced by mechanical occlusion on cellular components.

In this study we have investigated the possible alterations occuring in the intestinal ephitelium during occlusion. In particular we have investigated if, in ischaemic conditions, the damage implied possible modifications of: 1) enzymatic activity of some apical (maltase, leucin-aminopeptidase and alkaline phosphatase) and basolateral (Na+-K+-ATPase) membrane enzymes and 2) apical membrane K+ and Na+ permeability.

In order to measure enzymatic activity and Na+-K+-ATPase, homogenate from scraped intestinal mucosa and from brush-border membrane vesicles were used respectively.

In fact brush-border membranes can be isolated as vesicles ($_1$) and used to study ionic permeabilities and fluxes of several molecules(1-2).

MATERIAL AND METHODS

All chemicals of analytical grade, valinomycin, 3-3' diethylthiadicarbocyanine iodide (DiS-C2 (5)) and Wistar rats were purchased from the commercial source.

PREPARATION OF BRUSH-BORDER MEMBRANE VESICLES

Forty male and female Winstar rats (350-400 g) which had been fasted for 18-24 hours, were divided into two groups of twenty rats and were initially anaesthetized with 50 mg/Kg of Ketalar.

Then all animals underwent midline laparotomy and the last ileal loop was isolated (about 4 cm in length). The animals of the first group were used as a control; animals of the other group had the vascular peduncle strangulated and this situation was maintained for 60 minutes. All the animals were successively sacrificed previously taking the last isolated loop.

The intestinal segments were immediately excised, cut along its length and rinsed in ice-cold 0.9 % NaCl. Three gramms (fresh weight) of scraped intestinal mucosa (from 3 or 4

animals) were homogenized for three minutes in a blender, in a bottle containing 30 ml of 300 mM mannitol and 12 mM of (hydroxymethyl) aminomethane (Tris), adjusted to pH 7.1 with ethyleneglycol-bis ((-aminoethylether)-N,N'-tetra acetic acid (EGTA).

Just before starting the homogenization, 120 ml of cold distilled water were added to make the homogenization medium hyposmotic.

Five milliliters of this homogenate were taken for the enzymatic activity measurements: the rest of the homogenate was used to prepare brush-border membrane vesicles (BBMV) according to a Mg++-EGTA precipitation technique ($_{2}$), slightly modified.

The homogenate, after addition of 1.5 ml of 1.2 M MgCl2, was stored on ice for 15 minutes and centrifuged at 4,500 rpm for 15 mins; the resulting supernatant was centrifuged again at 18000 rpm for 30 minutes.

The pellet was resuspended in 35 ml of a solution containing 60 mM mannitol and 5 mM EGTA, adjusted to pH 7.4 with Tris.

A second Mg++ precipitation was performed as previously described with two centrifugation steps at 7,000 rpm for 15 min at 18,000 rpm for 30 min.

The resulting pellet was resuspended in a proper buffer for permeability experiments and centrifuged at 18,000 rpm for 30 mins. The final pellet (BBMV fraction) was resuspended in the same buffer by passing it 30 times through a fine-gauge needle. All procedures were carried out at 0-4 C°. Centrifugation were performed in a J2-21 Beckman centrifuge with a JA-20 rotor.

ENZYME ASSAYS

ENZYMATIC ACTIVITIES MEASUREMENTS

All enzymatic activities were measured in the starting homogenate.

The enzymatic activity of leucin-aminopeptidase was measured spectrophotometrically at 405 nm and 37 C° by using as substrate L-leucin-4-nitroanilide (LEUPA) and by measuring the absorbance of p-nitroaniline ($_{11}$).

The enzymatic activity of maltase was measured at pH 6.7 in the presence of 100 mM imidazol, 15 mM and 25 mM maltose; glucose production was assayed using the Sigma kit n° 16-UV.

The enzymatic activity of Na+-K+-stimulated ATPase was measured at 340 nm and 37 C° by following the redox reaction of NADH + H+/NAD+ (1). This activity was evaluated as the difference of ATPasic activity in the absence and in the presence of ouabain that inhibits completely the Na-K ATPase.

ISOLATION OF RAT BRUSH-BORDER MEMBRANE VESICLES

The isolation of rat intestinal BBMV was followed by the activity study of enzymes known to be charachteristic of different cellular organelles or components in the homogenate and in the final membrane fraction. Alkaline phosphatase (1), leucine peptidase (11) and maltase were measured as the marker enzymes for the BBM.

Maltase was measured at pH 6.7 in the presence of 100 mM imidazol, 15 mM MgCl2 and maltase; glucose production was assayed by the Sigma kit n° 16-UV.

The enrichment factors for these three marker enzymes (enzyme activities of final purified membrane pellet compared with those of the initial tissue homogenate) were 13, 14 and 11, respectively.

The methods used for evaluating enrichment factors for these enzymes were the same as described in the previous paragraph.

Na+-K+-stimulated adenosintriphosphatase (E.C. 3.6.1.3) was taken as the marker enzyme for the basolateral membranes.

Contamination by endoplasmic reticulum was monitored by analyzing KCN-insensitive NADH oxidoreductase activity (E.C. 1.6.99.2) in the presence of 0,9 mM KCN.

Succinate cytochrome c oxidoreductase activity (E.C. 1.6.99.1) was measured as the marker enzyme for mitochondria.

Negligible enrichment was found for marker enzymes of other cellular components, suggesting minimal contamination by basolateral or organelle membranes.

All the enzime assayes were carried out at 37 °C.

Protein concentration was measured by the Bio-Rad kit using lyophilized bovine plasma gamma-globulin as a standard.

All the measurements were carried out by with a III Trace

Beckman as spectrophotometer.

MEASUREMENTS OF FLUORESCENCE QUENCHING

Ionic permeability studies were carried out by using the fluorescent cyanine dye Dis-C2-(5) was measured with a Perkin Elmer LS-5 spectrofluorometer, equipped with an electronic stirring system and a thermostatized (37 °C) cuvette holder; fluorescence signals were continuously recorded (Hitachi-Perkin Elmer 561).

Excitation and emission wavelengths were 645 and 665 nm and both slit widths were set to 10 nm. 10 (l of a 0.6 mM dye solution in ethanol, 10 (l of a 0.89 mM valinomycin solution in ethanol (or ethanol only for control experiments), 1960 (l of a cuvette buffer were injected into a glass cuvette; valinimycin/protein ratio was 62 (g/mg and dye/protein ratio was 19.4 (g/mg.

The fluorescence value was set to 90 arbitrary fluorescence units and 20 (l (160 (g of protein) of suspension of vesicles were injected into the cuvette to start the experiment.

Intra- and extravesicular buffer had the same ionic stregth, pH, anion concentration and osmolarity.

RESULTS

ENZYMATIC ACTIVITY RATE

To establish the possibility of damage, during the ischaemic period, in intestinal epithelium, and its extent we compared the enzymatic activities (in the starting homogenate from the scraped mucosa) of enzymes leucin-aminopeptidase, alkaline phosphatase and maltase, (as enzymes strictly associated with the enterocyte basolateral membrane) both in control rats and in rats in which ischaemia was induced for one hour.

The measurement of the enzymatic activity of these enzymes was assumed as a parameter for the evaluation of the ischaemic damage due to the strangulation of the vascular peduncle.

The evaluation of the enzymatic activity in the homogenate, both in control and ischaemic rats, gave the results that were recorded on a panel in which we compare the enzymatic activities of leucin aminopeptidase, alkaline phosphatase, maltase and Na-K ATPase.

The enzymatic activity values for the same enzyme are different in different experiments: a possible explanation could be the different functional state of the intestinal mucosa in the single individuals, the different amount of the mucosal proteins from the scraped mucosa in the single experiment, etc.

Thus we compared, in every experiment, the enzymatic activities by referring enzymatic activities from ischaemic rats as the percent value of the enzymatic activity from control rats.

The averaged values (referred to n experiments) of the percentage values reffered to the control are reported below.

K+ PERMEABILITY

Brush-border membrane vesicles are a suitable system for studing metabolite transports. In fact they are enclosed as right-side-out vesicles and so the orientation of the membrane and of its enzymes is the same as that which occurs naturally.

These vesicles can also mantain an inside-negative membrane potential when the internal concentration of Na+and K+ is higher than the external.

DiS-C2(5) is fluorescent only when it is present outside the vescicles. On the contrary it is not fluorescent when it binds to the membrane (aspecific fluorescent quenching) or when it is distributed inside the membrane in response to an inside-negative menbrane potential (the dye is lipophylic and positively charged).

So, the more negative the inside membrane potential is, the more the external fluorescence decays. On the other hand, the width of membrane potential is directly correlated to:

1) The ion concentration gradient existing between the inside and the outside of the vesicles (artificially created);

2) Ion permeabilities which naturally exist in the membranes.

In order to generate an inside-negative membrane potential, vesicles were prepared in a buffer containing 100 mM KCl and diluted into a cuvette buffer to obtain an extravesicular KCl concentration of 1 mM.

Under these experimental conditions we can obtain four traces (a,b,c,d).

Vesicles prepared in the buffer were injected in the same buffer (trace a); the quenching of fluorescence depends on the distribution of the dye inside the lipidic bilayer (aspecific quenching) and not by membrane potential that, in these

conditions, is zero. Vesicles were then injected in a buffer containing 1 mM KCl (trace c); the quenching of fluorescence depends on the aspecific quenching and on the distribution of the dye inside the membrane due to the membrane potential created by K+ gradient (inside negative); in fact K+ can pass throught the membrane according to K+ permeability. When valinomicyn was added in the same conditions as trace a, trace c was obtained (aspecific fluorescence). When valinomycin was added in the same conditions as c, trace d was obtained; this signal depends on the aspecific fluorescence and on the membrane potential due to K+ gradient, but the presence of valinomycin causes K+ equilibrium across the membrane as valinomycin is a ionophore specific for K+. In these conditions we can obtain the maximal quenching of fluorescence; to this signal we can refer the signal obtained in trace c.

Under these experimental conditions by doing:

(trace c - trace a)/(trace d - trace b)

We can measure K+ permeability both in control and ischaemic vesicles. Assuming 100% value to K+ permeability in control vesicles, we can evaluate K+ permeability as percent value of the control vesicles.

The results of K+ permeability measurements carried out in the ischaemic rats are not reported here. However, K+ permeability increases 50% with respect to the control value.

DISCUSSION

The results of these experiments indicate:

1) specific activities of brush-border enzymes maltase, leucin-aminopeptidase, alkaline phosphatase decrease in the homogenate of the scraped mucosa from rats in which intestinal ischaemia was induced, with respect to the homogenate from control rats.

This means that the composition of the enzymatic pool of the homogenate from ischaemia and control rats is different. As specific activity of these enzymes remains almost unvaried for 24-48 hours (data not shown), the decreasing of enzymatic activity, observed after one hour of ischaemia, can be ascribed to the loss of plasmatic membranes or to the loss of whole cells induced by ischaemia this implies the decrease of enzymatic protein content, but not the decrease of their activity.

In particular, our findings about the decrease of activity of alkaline phosphatase(during the ischaemia) confirms the hypotesis of Rosato and coll. ([6]) who found that the intestinal pool of this enzyme (which is inhibited by l-phenylalanine) disappears from the seric enzyme pool;

2) we found that the enzymatic activity of he basololateral enzyme Na+-K+ ATPase is reduced to 71% of the control value; this reduction is smaller than that observed for the brush border enzymes; this means that a smaller amount of basolateral membranes, with respect to brush border membranes, is present in the starting homogenate and this can be due only to a smaller loss of basolateral membranes, with respect brush border membranes provoked by the ischaemic conditions. So we conclude that the greater damage induced by one hour of ischaemia is focalized above all on the apical membranes (brush border) rather than in the basolateral ones.

In conclusion, as enzymes are membrane proteins not directly connected to cellular metabolism and as their activity is correlated to the integrity of membrane itself, our results would indicate a loss of membrane enzymes due to 1) a loss of microvilli or 2) to a loss of epithelial cells.

These findings confirm the observed alterations of the microvillar organization which were demonstrated, even in 3'-5' minutes from the beginning of the ischaemia, means of electron microscopy technique or optic microscopy ([7,8]).

3) K+ permeability increases in brush border membrane vesicles from ischaemic rats with respect to control rats. This clearly indicates that, during the ischaemic period, an alteration of the organization of apical membranes occurred. The studies with the fluorescent dye, as an indicator of the ionic permeability, lead us to conclude that K+ permeability (that is considered as an index of functional integrity) is markedly altered and seems to indicate alterations of membrane structure and early damage (in one hour) induced by ischaemia in the structure of the brush border membranes ([10,12]). In fact K+ permeability increases 50% with respect to the control value. Since the amount proteins used in each experiment is the same in vesicles from control and ischaemic rats, the only possibility for a different change in fluorescence quenching is that brush border vesicles from ischaemic rats are more leaky than those from control rats. The reason why these vesicles are more leaky needs to be investigated, but the present point of view attributes it to the alteration of the lipidic bilayer structure, induced by

superoxide anion and hydroxyl radicals, an important role in the evolution of the ischaemic damage (10,12,[13,14]). This hypotesis is supported by the observation, in the same experimental model, of an increase of the extravascular peroxydase activity that is strictly connected with the removal of superoxide radicals.

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