Activation and release of proteolytic kinin-forming enzymes from rat jejunal loops perfused with hyperosmolar glucose solutions

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SUMMARY Loops of rat jejunum were perfused *in vivo* for one hour with de Jalon solutions of increasing glucose concentration. The esterolytic activity of the perfusate and of the extracted loop was measured using benzoyl arginine ethyl ester (BAEe) as substrate. The enzymes lactate dehydrogenase, isocitrate dehydrogenase, and acid phosphatase were measured in the perfusate to estimate the degree of cellular damage. There was a significant depletion of kallikrein from the intestine and a raised level in the perfusate during the hyperosmolar perfusions. No significant difference in the kallikrein levels was observed between the normal jejunum and the iso-osmolar perfused jejunum. Prolonged contact with hyperosmolar solutions caused some cellular damage but this effect appeared to be distinct from the release of kallikrein, which occurred very rapidly during the first 20 minutes. These results confirm that hyperosmolar intestinal contents result in the release of kinin-forming proteolytic enzymes into the circulation and suggest that this mechanism is involved in the syndrome of vasomotor dumping after gastric surgery.

The presence of kinin-forming enzymes in the gastrointestinal tract was first demonstrated by Amundsen and Nustad (1965) in cell homogenates. Since then kininogenases (kinin-forming enzymes) have been identified in the gastrointestinal tract of various species including man (Zeitlin, 1970; Seki *et al.*, 1972) and the activation and release of these enzymes have been implicated in the pathology of some gastrointestinal disorders such as the carcinoid and the post-gastrectomy vasomotor dumping syndromes.

Release of vasoactive kinin peptides have been demonstrated during attacks of vasomotor dumping (Zeitlin and Smith, 1966; MacDonald *et al.*, 1969; Cuschieri and Onobanjo, 1974; Wong *et al.*, 1974; Cuschieri and Worthington, 1976) and this disorder is known to be associated with rapid emptying of the stomach remnant, resulting in hyperosmolar intestinal contents. The *in vitro* study reported by Zeitlin (1970) showed that activation and release of intestinal kallikrein results from the incubation of fragments of jejunal mucosa in hyperosmolar solutions and suggested hyperosmolar damage to the intestinal mucosal cells as a possible mechanism for

the release of potent proteolytic enzymes. The present study was designed to test this hypothesis in the *in vivo* situation.

Methods

RAT PERFUSION

Wistar rats (weight range 310-530 g) were anaesthetised with intraperitoneal nembutal (60 mg/ml, 0.1 ml/g) and then mounted on a heated operating table (39°C). The abdomen was shaved and a small midline incision made through the anterior abdominal wall. A segment of small intestine was selected at a fixed distance from the duodenojejunal flexure (5.0 cm). The selected loop was transected at either end. Two silicone rubber cannulae were inserted at either end and secured in place. The restoration of the continuity of the gastrointestinal tract was effected by an end-to-end anastomosis. The isolated loop was left in the abdominal cavity, which was closed and the two silicone rubber cannulae brought out through the wound at either end. The length of the isolated loop measured along its antimesenteric border was 9.71 ± 0.84 cm. The loop was then perfused at 39°C with one of the de

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Table 1 Concentrations and osmolarity of various de Jalon solutions

Perfusate	m osmol		Glucose mM	NaCl mM	KCl mM	NaHCO ₃ mM	CaCl mM
	$\bar{x}$	SD					
De Jalon-1	259.6	25.39	2.77	154	5.6	5.95	0.27
De Jalon-2	534.9	46.97	250	154	5.6	5.95	0.27
De Jalon-3	671.8	5.01	334	154	5.6	5.95	0.27
De Jalon-4	1358.4	84.8	1000	154	5.6	5.95	0.27

Jalon solutions shown in Table 1 at a rate of 30.0 ml per hour. The type of de Jalon solution perfused in terms of osmolarity through a given loop was completely randomised. The perfusion was continued for 60 minutes and samples of effluent taken every 20 minutes. At the end of the experiment the perfused loop was excised and the animal killed. The removed loop was then washed in saline, blotted, and weighed. It was minced with scissors and homogenised in glass-distilled water (W/V: 1/10) using a Potter Elvjham-type homogeniser. This was centrifuged at $12\,000\,g \times 2\,h$ in an MSE 25 high-speed centrifuge and the supernatant removed and deep frozen for subsequent assay.

The first 10 samples of extracted loops were treated differently. Before centrifugation each was divided into three equal parts. One third was centrifuged immediately, one was left to autolyse at room temperature overnight, and one was heated at 55°C for 10 minutes.

BIOCHEMICAL MEASUREMENTS

Estimation of esterolytic activity

This was measured using the benzoyl arginine ethyl ester (BAEe), alcohol dehydrogenase linked method as previously described by Worthington and Cuschieri (1974, 1976). In some cases, however, the activity was very low and the method was modified slightly. A sample of the extracted loop (0.2 ml) or perfusate (0.5 ml) was incubated in a cuvette for 15 minutes at 37°C with BAEe (0.2 mg) and buffer to give a final volume of 2.85 ml. Alcohol dehydrogenase (0.6 mg) and NAD⁺ (1.5 mg) were then added and the extinction at 366 nm recorded continually for two minutes. In each case a blank containing no BAEe was used. The spontaneous breakdown of BAEe at this pH was measured using identical conditions but omitting the enzyme. The effects of the inhibitor Trasylol (1 000 KIU) and the inhibitors from ovomucoid and soybean (1.0 mg) were determined. These were added to the mixture in the cuvette before incubation at 37° $\times$ 15 min. Each estimation was performed in triplicate.

Measurement of lactate dehydrogenase, isocitrate dehydrogenase (NADP linked) and acid phosphatase The Boehringer Mannheim GMBH Biochemical Test combination kits were used to assay these enzymes. In each case the amount of serum specified was substituted by a similar amount of perfusate.

Other Estimations

Protein in the perfusate samples was assayed using the method of Lowry *et al.* (1951) and in the extracted loop samples by the Biuret method as described by Gornall *et al.* (1949). Bovine serum albumin was used as a standard.

Glucose was measured using the glucose hexokinase Boehringer Mannheim GMBH Biochemical Test combination kit. Sodium and potassium were measured using an EEL 450 flame photometer, the osmolarity of the de Jalon solutions was measured using an Osmette S osmometer, and haemoglobin was estimated using Hemastix reagent strips (Ames Co.).

Results

Deep freezing the samples did not cause any loss in enzyme activity if assayed within one month. During the perfusion there was no significant change in the concentration of glucose, sodium, and potassium or the volume of the perfusate. The esterolytic activity was calculated both as international milliunits per ml (ImU/ml) and per mg protein (ImU/mg). The latter value did not appear to be relevant, probably because of a large amount of extraneous protein being present. Neither autolysis nor heating increased the esterolytic activity per ml of the extracted loop. Both had the effect of reducing the protein content of the extract by as much as 36 to 57%, making the specific enzyme activity much higher. However, from the argument mentioned above and from the fact that the decrease in protein content was variable, thus introducing a greater error when comparing the results, all further work was carried out on the untreated material.

The mean values and standard deviations for esterolytic activity are shown in Table 2 and for the other enzymes in Table 3. For the perfusate these values are for the pooled samples from each experiment. However, the esterolytic activity was consistently highest during the first 20 minutes, whereas the increase in the concentration of the other enzymes occurred during the last 20 minutes of the perfusion.

The raw data for all the enzyme activities was shown to have a log₁₀ distribution and for the purpose of Student's *t* test logarithmic transformation of the data was carried out. The *P* values obtained after

Table 2 Mean values and standard deviations for esterolytic activity (ImU/ml) of extracted loop and perfusate

Group	n	Extracted loop		Perfusate	
		$\bar{x}$	SD	$\bar{x}$	SD
Normal unperfused	10	98.90	42.68	—	—
De Jalon-1 perfused	10	73.74	26.29	7.88	9.86
De Jalon-2 perfused	10	46.84	23.29	28.01	27.6
De Jalon-3 perfused	10	53.29	21.86	16.83	17.73
De Jalon-4 perfused	10	49.99	26.63	19.87	19.83

Table 3 Mean values for isocitrate dehydrogenase (ICDH), lactate dehydrogenase (LDH), and acid phosphatase of perfusate (expressed as ImU/ml)

Group	n	ICDH		LDH		Acid phos.	
		$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
De Jalon-1 perfused	10	2.86	2.72	57.04	48.89	2.39	1.68
De Jalon-2 perfused	10	8.28	8.69	268.69	174.61	3.12	4.27
De Jalon-3 perfused	10	2.85	2.34	307.24	219.70	2.04	1.65
De Jalon-4 perfused	10	10.59	8.5	229.22	175.32	3.76	4.52

performing the *t* test for unpaired data are shown in Tables 4 and 5. The test was performed between all the groups but only those in which a significant difference was observed are included in the Tables.

The normal unperfused loop contained the highest esterolytic activity and this was significantly higher than the activity in the loops perfused with hyperosmolar solutions. The esterolytic activity of the de Jalon-1 perfused loop was not significantly lower than the normal group but it was significantly higher than the other perfused loops.

In the perfusate the esterolytic activity was lowest in the de Jalon-1 group and was significantly raised in the de Jalon-2 and 4 groups but not in 3. Of the three other enzymes studied, lactate dehydrogenase was the only one that was raised during all the hyperosmolar perfusions; isocitrate dehydrogenase was raised with de Jalon-4 solution only.

The correlation coefficient between esterolytic activity and the various other values are shown in Table 6. The only significant correlation appeared to be between isocitrate dehydrogenase and esterolytic activity, and acid phosphatase and esterolytic activity in the perfusate. There was no correlation between the amount of haemoglobin and any of the enzymes.

Trasylol almost completely inhibited the esterolytic activity of both the extracted loop ($91.5 \pm 10.7\%$) and the perfusate ($88.7 \pm 15.6\%$). The inhibition by soybean and ovomucoid was much more variable, the mean values being $35.6 \pm 16.8\%$ and $17.1 \pm 10.3\%$ respectively for the extracted loop. These

Table 4 Probability values (*P*) for Student's *t* test for significance for $\log_{10}$ values of esterolytic activity

Group	Extracted loop		Perfusate De Jalon-1 perfused
	Normal Unperfused	De Jalon 1 perfused	
De Jalon-1 perfused	0.1	—	—
De Jalon-2 perfused	0.005	<0.02	0.05
De Jalon-3 perfused	<0.001	<0.01	>0.1
De Jalon-4 perfused	<0.005	<0.05	0.05

Table 5 Probability values (*P*) for Student's *t* test for significance between $\log_{10}$ values of enzyme activities for de Jalon 1 perfused group and other groups

	Perfusate		Extracted loop	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
Glucose	0.0318	0.8	0.227	0.2
Esterolytic act. loop	0.1670	0.1		
ICDH	0.4248	0.01		
LDH	0.2683	0.05		
Acid phos.	0.3499	0.05		

Table 6 Correlation coefficient (*r*) and probability values (*P*) for esterolytic activity of perfusate and extracted loops and various other values

Group	ICDH	LDH	Acid phos.
De Jalon-2 perfused	>0.05	<0.001	0.4
De Jalon-3 perfused	0.7	<0.001	0.5
De Jalon-4 perfused	<0.01	<0.01	0.99

inhibitors were not used with the perfusate because of insufficient samples.

Discussion

The three enzymes lactate dehydrogenase, isocitrate dehydrogenase, and acid phosphatase were measured in an attempt to estimate the amount of cellular disruption occurring during the perfusions. The enzymes lactate dehydrogenase and isocitrate dehydrogenase (NADP linked) are thought to occur predominantly in the soluble fraction and acid phosphatase in the lysosomal fraction of the cell (Dixon and Webb, 1962; de Duve *et al.*, 1962). During all the hyperosmolar perfusions the significantly raised levels of lactate dehydrogenase indicated that a certain degree of cellular damage had occurred. However, this appeared to be an all-or-none relationship, there being no further release of this enzyme on increasing the osmolarity above 530 mosmol.

Isocitrate dehydrogenase, which is also a soluble enzyme, did not follow the same pattern, although the level in the de Jalon-4 perfused group was

significantly higher than the level in the de Jalon-1 group. From the values in Table 3, it can be seen that there is no relationship between the amount of isocitrate dehydrogenase released and the osmolarity or glucose concentration of the perfusate. It is therefore not possible to use this enzyme as an index of cellular damage caused by high glucose concentrations. The level of acid phosphatase did not significantly change during the perfusion, indicating that little lysosomal damage had occurred.

The esterolytic activity found in the perfusate during the hyperosmolar perfusions was raised, although not significantly so, in the de Jalon-3 group. The results differ from those observed by Zeitlin (1970) who showed a progressive increase in the release of kallikrein into the incubate with increasing osmolarity. However, the conditions of this experiment differed considerably from our own. The work was carried out with small prisms of tissue (0.4 mm) which did not represent the true *in vivo* situation where, in fact, one would expect the enzyme kallikrein to be released into the blood stream to act upon its plasma substrate, kininogen. Therefore, the release into the lumen of the intestine *in vivo* should not be considerable. A better indication would be in the depletion of kallikrein from the intestine itself and this is what the present results indicate. The findings of the present study show that activation of kallikrein occurs when loops of intestine are perfused with solutions with an osmolarity outside the normal physiological range. However, no further increase in the release of proteolytic activity was found when the osmolarity of the perfusate was increased from 530 to 1 350 mosmol. Like lactate dehydrogenase, the release of proteolytic enzymes in response to perfusion with hyperosmolar solutions seems to be an all-or-none phenomenon.

The release of kallikrein into the perfusate was rapid, occurring in the first 20 minutes of the perfusion, whereas cellular damage as indicated by lactate dehydrogenase occurred only after 40 minutes of perfusion. Therefore, the effect of hyperosmolar solutions on the release of proteolytic enzymes appears to be specific and may not be ascribed directly to cellular damage.

The correlation observed between the release of proteolytic activity and the two enzymes isocitrate dehydrogenase and acid phosphatase, while in no way related to the glucose concentration, may be due to some of the jejunal membranes being more permeable to the passage of enzymes than others. The lack of correlation between any of the enzymes and the haemoglobin content rules out the possibility that this relationship is due to enzymes from the blood rather than the jejunum.

The inhibition profile for the enzyme extracted

from the jejunal loop indicates that approximately 17% of the enzyme activity was due to plasma kallikrein or trypsin, 8% due to plasmin, and the remaining 75% of the activity due to intestinal kallikrein (Vogel and Werle, 1970; Zeitlin, 1972). As these tissues were not blood free, one would expect some contamination with plasma enzymes. There was no significant difference between the inhibition patterns of each group.

The effects of hyperosmolar solutions upon the rat jejunum *in vivo*, when projected to the human situation, reinforce the findings of various workers on the role of the kinin-kallikrein system in the dumping syndrome. In this syndrome the rapid emptying of the stomach remnant leads to a situation where the intestinal contents are hyperosmolar. This study has shown that hyperosmolar intestinal contents can act as a mechanism for the release of intestinal kinin-forming enzymes such as kallikrein. The findings from this study have, also, practical implications with regard to enteral hyperalimentation as most elemental diets are hyperosmolar.

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