

# Release of vasodilator, but not vasoconstrictor, neuropeptides and of enteroglucagon by intestinal ischaemia/reperfusion in the rat

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## Abstract

**Reperfusion of ischaemic intestine is characterised by an initial hyperaemia with ensuing mucosal repair. This study investigated possible roles for gut vasoactive neuropeptides and trophic peptides in these phenomena. Groups of rats were monitored during superior mesenteric artery occlusion for five or 20 minutes, with or without subsequent reperfusion for five minutes. Peptide concentrations (fmol/ml) in arterial blood, were measured using specific radioimmunoassays. Intestinal ischaemia alone did not cause haemodynamic disturbance or peptide release. Reperfusion, after five minutes of ischaemia, resulted in arterial hypotension and a rise in plasma vasoactive intestinal polypeptide (mean (SEM)) (37 (3), control 11 (4),  $p < 0.001$ ). After 20 minutes of ischaemia, reperfusion resulted in greater hypotension ( $p < 0.05$ ) and release of both vasoactive intestinal polypeptide (31 (3),  $p < 0.05$  *v* control) and the more potent vasodilator  $\beta$ -calcitonin gene related peptide (49 (3), control 23 (1),  $p < 0.001$ ). By contrast, the vasodilators  $\alpha$ -calcitonin gene related peptide and substance P and the vasoconstrictors neuropeptide Y, peptide YY, and somatostatin were not released. Bombesin, a stimulatory neuropeptide, was released after 20 minutes of ischaemia/reperfusion (13 (2), control 7 (3),  $p < 0.05$ ). Plasma enteroglucagon rose from control (51 (4)) to 110 (16) ( $p < 0.001$ ) and to 158 (27) ( $p < 0.005$ ) after five and 20 minutes of ischaemia/reperfusion. The potent enteric vasodilators vasoactive intestinal polypeptide and  $\beta$ -calcitonin gene related peptide, unopposed by vasoconstrictors, may promote post-ischaemic intestinal hyperaemia. The rise in plasma enteroglucagon may point to diffuse mucosal injury and is consistent with the putative trophic role of this peptide.**

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Reperfusion of the ischaemic intestine is characterised by an initial reactive hyperaemia.<sup>1,2</sup> Several vasodilator metabolites, released from the ischaemic intestine, have been proposed as potential mediators of this response.<sup>1,3</sup> In addition, the intestine is furnished with a rich supply of nerves containing both vasodilator and vasoconstrictor

peptides.<sup>4-6</sup> These nerves constitute an abundant perivascular network,<sup>4</sup> thereby implicating vasoactive peptides in the regulation of intestinal blood flow.<sup>4,6</sup>

Vasoactive intestinal polypeptide (VIP) is a potent vasodilator neuropeptide<sup>7,8</sup> whose release during intestinal ischaemia/reperfusion is well reported.<sup>9-12</sup> More recently, calcitonin gene related peptide (CGRP) has also been identified as a most powerful vasodilator neuropeptide.<sup>13,14</sup> CGRP is abundantly distributed in perivascular nerves of the gut<sup>15,16</sup> and has an important role in the regulation of intestinal blood flow.<sup>15</sup> It exists in two different forms,  $\alpha$ -CGRP and  $\beta$ -CGRP, which are equipotent in their vasodilatory and other actions.<sup>17</sup> In the intestine,  $\beta$ -CGRP exists, in intrinsic enteric neurons, in much greater concentrations than  $\alpha$ -CGRP, which is found exclusively in extrinsic nerves.<sup>17</sup> Substance P is another intrinsic enteric vasodilator neuropeptide,<sup>18,19</sup> which may have a role in ischaemic vasodilatation.<sup>3</sup>

Neuropeptide Y is a vasoconstrictor,<sup>20</sup> which is widely distributed in intestinal perivascular nerves.<sup>4,5,21</sup> In a previous study there was no release of neuropeptide Y from the reperfused ischaemic intestine.<sup>11</sup> By contrast, peptide YY, a vasoconstrictor peptide of mucosal cell origin<sup>22</sup> was released during ischaemia/reperfusion.<sup>11</sup> Somatostatin has numerous actions, including vasoconstriction, and is distributed in both enteric nerves and mucosal endocrine cells.<sup>23</sup>

The purpose of this study was to determine the responses of these powerful splanchnic vasoactive peptides to intestinal ischaemia/reperfusion. We hypothesised that, in line with the observed reactive hyperaemia of reperfusion, there is preferential release of vasodilator, but not vasoconstrictor peptides, which could be detected by increases in their peripheral plasma concentrations. These agents may influence intestinal blood flow and oxygenation during a critical period in early reperfusion.

Intestinal ischaemia/reperfusion is not characterised solely by vascular changes. The mucosa also suffers an injury proportional to the severity and duration of the ischaemic insult.<sup>24</sup> In this study we hypothesised that enteroglucagon, an intestinal mucosal peptide with a putative trophic role<sup>25,26</sup> is released into the circulation during reperfusion to initiate mucosal repair. Bombesin, a neuropeptide<sup>27</sup> with widespread stimulatory actions, induces release of enteroglucagon<sup>25,28</sup> and of several neuropeptides<sup>6,28</sup> and may antagonise the

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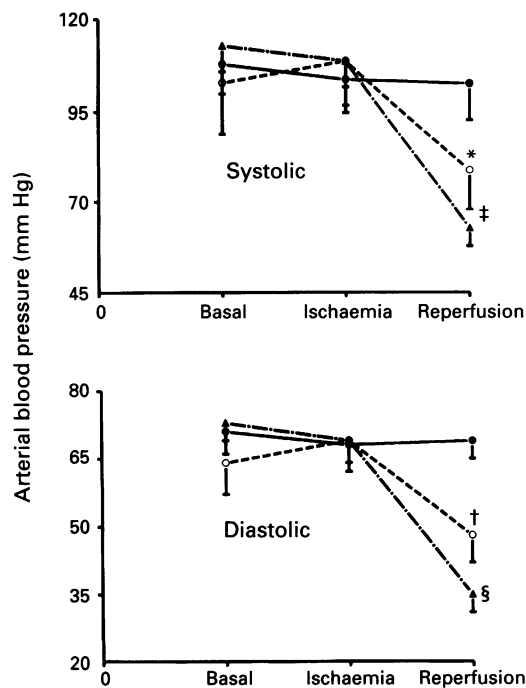


Figure 1: Effect of ischaemia/reperfusion on arterial blood pressure in control (●—●), 5 I/R (○---○), and 20 I/R (▲---▲) animals. Responses in the 5 I and 20 I animals were similar to control and therefore not shown for reasons of clarity. \* $p < 0.01$  v control, † $p < 0.05$  v control, ‡ $p < 0.001$  v control and <0.005 v 5 I/R, § $p < 0.001$  v control and <0.05 v 5 I/R.

actions of somatostatin.<sup>6</sup> The second aim of this study was, therefore, to monitor the plasma release of enteroglucagon and bombesin in response to intestinal ischaemia/reperfusion.

### Methods

Five groups of male Wistar rats ( $n=6$  per group), weighing 280–320 g, deprived of food for 12 hours but permitted free access to water, were studied. Animals were anaesthetised with an intraperitoneal injection of a mixture of fentanyl 0.2 mg, fluanisone 5 mg, midazolam 3 mg, and allowed to breathe spontaneously. Further doses of anaesthetic were given during the experiment as required. Animals were placed on a thermal blanket to ensure a constant body temperature between 37°C and 38°C.

### EXPERIMENTAL PROTOCOL

A cannula, inserted into the lower abdominal aorta through the left femoral artery, was connected to a PT400 pressure transducer (Palmer Bioscience, Sheerness, Kent, England) for continuous monitoring of the arterial blood pressure and heart rate.

TABLE 1 Plasma concentrations of peptides unaffected by ischaemia/reperfusion

Groups	$\alpha$ -CGRP	Substance P	Neuropeptide Y	Peptide YY	Somatostatin
Control	23 (3)	15 (2)	90 (22)	31 (3)	25 (7)
5 I	21 (4)	13 (3)	85 (15)	34 (9)	24 (7)
20 I	25 (4)	17 (3)	90 (25)	26 (7)	16 (2)
5 I/R	29 (5)	19 (2)	150 (43)	47 (7)	37 (12)
20 I/R	36 (3)	21 (2)	119 (26)	35 (5)	25 (4)

Plasma peptides are mean (SEM) fmol/ml.

Heparinised saline (100 U/ml) was infused (10 ml/kg/h) through a cannula inserted in the left femoral vein, to prevent dehydration during the experiment. Through a midline laparotomy the superior mesenteric artery was exposed at its origin, taking care to preserve the perivascular nerves. After a period of stable baseline observation most of the small bowel was rendered ischaemic by occluding the superior mesenteric artery with a small bulldog clamp applied to its origin. Two experimental groups were subjected to ischaemia alone, without reperfusion, for five (5 I) or 20 (20 I) minutes. In the other two experimental groups the ischaemic bowel was allowed to reperfuse for five minutes by releasing the superior mesenteric artery clamp after 5 (5 I/R) or 20 (20 I/R) minutes of occlusion. Control animals underwent laparotomy and sham superior mesenteric artery occlusion. Aortic blood was obtained from the femoral artery cannula in the last two minutes of the experiment, thus killing the animals by exsanguination.

### RADIOIMMUNOASSAYS

Blood samples were collected in lithium heparin tubes, containing 4000 KIU of aprotinin (Trasylol, Bayer Limited, UK), and centrifuged immediately at 2500 rpm for 10 minutes. The plasma was decanted and stored at  $-20^{\circ}\text{C}$  until assay. Plasma concentrations of VIP,<sup>29</sup>  $\alpha$ - and  $\beta$ -CGRP,<sup>17</sup> substance P,<sup>29</sup> neuropeptide Y,<sup>30</sup> peptide YY,<sup>31</sup> somatostatin,<sup>29</sup> bombesin,<sup>29</sup> and enteroglucagon<sup>25</sup> were determined using well established sensitive and specific radioimmunoassays.

### DATA PRESENTATION AND ANALYSIS

Arterial blood pressure (mm Hg) is presented as mean (SEM). The incremental changes, from baseline, at the end of the ischaemic and reperfusion periods, or equivalent time points in the control and non-reperfused ischaemic animals, were analysed by one way analysis of variance (ANOVA). Plasma peptide concentrations (mean (SEM), fmol/ml) were also compared, between groups, by ANOVA. When significant overall changes were found, Student's two tailed  $t$  tests were applied to individual values.

### Results

#### ARTERIAL BLOOD PRESSURE AND HEART RATE

There were no significant changes with intestinal ischaemia alone in any of the groups, but reperfusion resulted in significant changes in systolic and diastolic blood pressure (Fig 1). A significant fall in systolic blood pressure occurred in the 5 I/R (79 (11) mm Hg) and 20 I/R (63 (5) mm Hg) groups compared with control (103 (10) mm Hg),  $p < 0.01$  and  $p < 0.001$ , respectively. Similarly diastolic pressure was significantly reduced in the 5 I/R (48 (6) mm Hg) and the 20 I/R (35 (4) mm Hg) animals compared with controls (69 (4) mm Hg),  $p < 0.05$  and  $p < 0.001$ ,

TABLE II Plasma concentrations of peptides changed by ischaemia/reperfusion

Groups	VIP	$\beta$ -CGRP	Bombesin	Enteroglucagon
Control	11 (4)	23 (1)	7 (3)	51 (4)
5 I	7 (2)	35 (7)	7 (3)	55 (12)
20 I	5 (2)	26 (3)	7 (2)	43 (7)
5 I/R	37 (3)	34 (6)	10 (4)	110 (16)
20 I/R	31 (3)	49 (3)	13 (2)	158 (27)

Plasma peptides are mean (SEM) fmol/ml.

respectively. The fall in systolic ( $p < 0.005$ ) and diastolic ( $p < 0.05$ ) blood pressure in the 20 I/R was significantly greater compared with the 5 I/R animals. There was no overall change in heart rate with either ischaemia alone, or during reperfusion.

#### PLASMA PEPTIDES

The plasma concentrations of  $\alpha$ -CGRP, substance P, neuropeptide Y, peptide YY, and somatostatin did not change significantly in any of the experimental groups, compared with control (Table I). By contrast, there were significant changes in the plasma concentrations of VIP,  $\beta$ -CGRP, bombesin, and enteroglucagon. Table II shows that significant increases in the plasma peptides occurred only as a result of ischaemia/reperfusion and not with ischaemia alone. Plasma VIP (Fig 2) nearly trebled in the 20 I/R and more than trebled in the 5 I/R animals. Similarly, enteroglucagon (Fig 3) increased by more than two and three times in the 5 I/R and 20 I/R groups, respectively. Plasma concentrations of  $\beta$ -CGRP (Fig 4) and bombesin (Fig 5) roughly doubled in the 20 I/R animals. In contrast with VIP and enteroglucagon, however, there were no increases in plasma  $\beta$ -CGRP or bombesin in the 5 I/R animals.

Strong correlations were shown between the plasma concentrations of bombesin and enteroglucagon ( $r = 0.76$ ), and of VIP and enteroglucagon ( $r = 0.71$ ). In addition, good correlations were obtained between all the peptides whose plasma concentrations changed as a result of ischaemia/reperfusion (Table III). There was also a strong correlation, however, between  $\alpha$ - and  $\beta$ -CGRP ( $r = 0.72$ ) and a weaker correlation between VIP and  $\alpha$ -CGRP ( $r = 0.43$ ) despite the lack of overall change in  $\alpha$ -CGRP concentrations.

#### Discussion

In this study we report considerable arterial

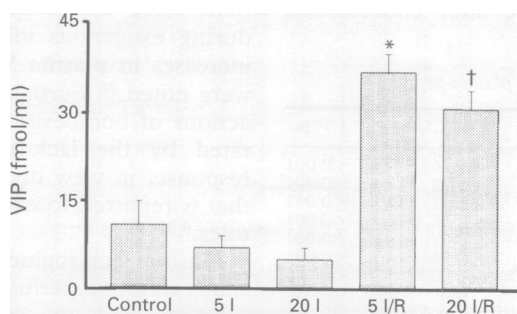


Figure 2: Plasma VIP concentrations in control, ischaemia alone, and ischaemia/reperfusion groups. \* $p < 0.001$  v control, † $p < 0.005$  v control.

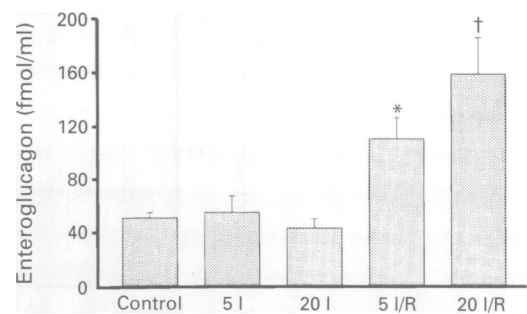


Figure 3: Plasma enteroglucagon concentrations in control, ischaemia alone, and ischaemia/reperfusion animals. \* $p < 0.01$  v control, † $p < 0.005$  v control.

hypotension and a distinct pattern in the profile of circulating gut peptides during reperfusion of the ischaemic rat intestine. Hypotension associated with reperfusion has been reported previously, after ischaemic intervals of 15<sup>9</sup> and 20<sup>10</sup> minutes, in agreement with our findings. In this study reperfusion even after a brief ischaemic insult (five minutes) resulted in significant hypotension. Reactive hyperaemia, manifest by vasodilatation<sup>1,2,12</sup> and increased intestinal blood flow,<sup>10,32</sup> occurs once a superior mesenteric artery occlusion is released. A number of potential mediators participate in these responses providing a link between tissue anoxia and arteriolar vasodilatation.<sup>1-3</sup>

We now report that release of two potent vasodilator gut neuropeptides, VIP<sup>7,8</sup> and  $\beta$ -CGRP<sup>13-15</sup> coincided with the hypotension of reperfusion. This study confirms previous reports of VIP involvement in post-ischaemic vasodilatation<sup>9-12</sup> and suggests that VIP neurons may be highly sensitive to intestinal ischaemia as equivalent plasma responses occurred after each ischaemic interval. We have also shown that prolonged ischaemia is associated with release of the more potent vasodilator  $\beta$ -CGRP,<sup>15</sup> corresponding to the development of more profound hypotension. Histamine and adenosine, which are released from the ischaemic intestine<sup>2,33</sup> and have been proposed as vasodilators in reactive hyperaemia,<sup>2,3</sup> are not as potent as VIP<sup>7</sup> and CGRP.<sup>13</sup> Therefore, release of these neuropeptides during reperfusion conforms with the hypothesis that they may also be involved in post-ischaemic vasodilatation.

The plasma concentrations of VIP and  $\beta$ -CGRP rose in the first few minutes of reperfusion, but as portal venous samples were not

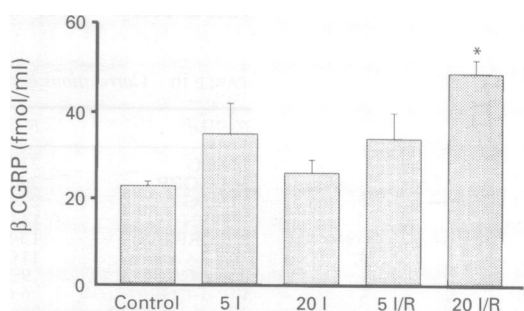


Figure 4: Plasma  $\beta$ -CGRP concentrations in control, ischaemia alone, and ischaemia/reperfusion animals. \* $p < 0.001$  v control.



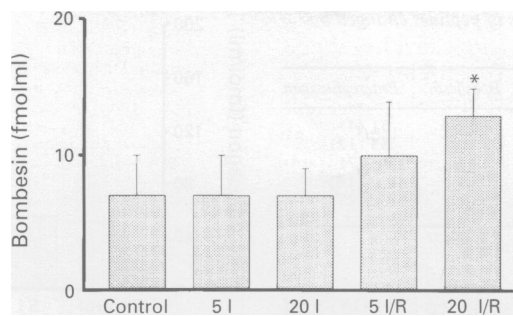


Figure 5: Plasma bombesin concentrations in control, ischaemia alone, and ischaemia/reperfusion animals. \* $p < 0.05$  v control.

obtained, their tissue of origin cannot be precisely determined. The intestine is, however, by far the richest source of these neuropeptides<sup>4-6 16 17</sup> and raised concentrations of VIP have been reported in the portal vein during intestinal ischaemia.<sup>10-12</sup> The neuropeptides probably accumulated in the ischaemic intestine and were washed out into the peripheral circulation during reperfusion. Therefore these putative neurotransmitters,<sup>8 14</sup> located in enteric primary autonomic neurons,<sup>4 5 17</sup> could be released during ischaemia by a local axon reflex mechanism after activation of chemosensitive mucosal receptors.<sup>18 34</sup> This seems a more plausible explanation than speculating peptide release from an extraintestinal source, secondary to systemic hypotension. Thus, if present in the ischaemic gut at the onset of reperfusion, they may be implicated in important pathophysiological events. These powerful mesenteric vasodilators<sup>8 15</sup> facilitate tissue perfusion and potentiate fluid and protein extravasation.<sup>35 36</sup> Consequently, they may promote the generation of oxygen free radicals by xanthine oxidase<sup>24</sup> and foster an environment favourable to neutrophil activation, leading to reperfusion injury.<sup>37</sup> Although these actions must remain speculative both VIP and  $\beta$ -CGRP are active at the plasma concentrations measured here<sup>13 14 38 39</sup> and much higher concentrations can be achieved at neuroeffector junctions.<sup>8 14 38</sup>

In this study the plasma concentrations of the other mesenteric vasodilator neuropeptides  $\alpha$ -CGRP and substance P did not rise. In view of the observed correlation between  $\alpha$ - and  $\beta$ -CGRP, however, it is possible that  $\alpha$ -CGRP was also released from perivascular nerves<sup>15 16</sup> but remained tightly bound to tissue receptors.<sup>14</sup> Substance P, another putative intestinal vasodilator<sup>3 18 19</sup> is active after endoluminal

release,<sup>40</sup> which might explain why its plasma concentrations did not rise. Alternatively, differences in tissue distribution and concentration of these neuropeptides may account for the discrepancies in their plasma responses. Within the enteric nervous system VIP is the most abundant neuropeptide<sup>4-6</sup> and there is a much larger population of intrinsic  $\beta$ -CGRP compared with extrinsic  $\alpha$ -CGRP neurones.<sup>17</sup> There is also a far richer supply of CGRP in mesenteric vascular nerves compared with substance P<sup>16</sup> whose neurons are mainly distributed in the myenteric plexus<sup>6</sup> where they may be protected from ischaemia. By contrast, VIP and  $\beta$ -CGRP neurons are abundant in the mucosa/submucosa, regions susceptible to the effects of ischaemia.<sup>24 37</sup>

Plasma peptide YY, a potent splanchnic vasoconstrictor peptide,<sup>22</sup> remained unaffected by intestinal ischaemia/reperfusion. As peptide YY is mainly found in colonic mucosal cells,<sup>22 41</sup> its previously reported release may have resulted from prolonged ischaemic injury to the distal bowel.<sup>11</sup> Neuropeptide Y, a potent vasoconstrictor neuropeptide<sup>20</sup> of intrinsic and extrinsic enteric nerves,<sup>21</sup> was not released from the reperfused intestine in agreement with the earlier study.<sup>11</sup> Plasma concentrations of somatostatin, another vasoconstrictor neuropeptide evenly distributed throughout the small bowel,<sup>23</sup> did not rise either. The lack of vasoconstrictor response contrasted sharply with the rise in plasma vasodilators, suggesting an active release process for VIP and  $\beta$ -CGRP rather than non-specific passive leakage from anoxic nerve endings. Although the liver is not the major site of peptide metabolism,<sup>42</sup> it is possible that hepatic extraction of vasoconstrictors obscured a rise in their plasma concentrations. Indeed, a proportion of portal venous somatostatin is cleared by the liver but the efficiency of this mechanism may be impaired by acidosis<sup>43</sup> in ischaemia/reperfusion. Furthermore, different stimuli can release peptide YY, neuropeptide Y, and somatostatin from the splanchnic region into the peripheral circulation<sup>30 31 44</sup> thus justifying the use of peripheral plasma for peptide measurement as presently used.

Plasma concentrations of the gut neuropeptide bombesin<sup>27</sup> increased during reperfusion especially after an ischaemic interval of 20 minutes, and correlated significantly with VIP and  $\beta$ -CGRP, consistent with the actions of bombesin in modulating the release of several peptides.<sup>6</sup> Bombesin plasma concentrations were comparable with those achieved during exogenous infusion, when significant increases in plasma VIP and enteroglucagon were noted.<sup>28</sup> Furthermore, in this study, the actions of bombesin should have been facilitated by the lack of plasma somatostatin response, in view of the antagonistic relation that is reported to exist between the two peptides.<sup>6 25 28</sup>

Plasma enteroglucagon increased significantly during reperfusion, after each ischaemic interval, and its correlation with plasma bombesin is in line with the proposed role of bombesin in its release.<sup>25 28</sup> The correlations of

TABLE III Correlations between plasma peptides

Peptides	F ratio	r Value	p Value
BN-EG	21.7	0.76	<0.001
$\alpha$ - $\beta$ -CGRP	30.9	0.72	<0.001
VIP-EG	27.9	0.71	<0.001
VIP-BN	14.0	0.58	<0.001
$\beta$ -CGRP-EG	13.5	0.57	<0.001
VIP- $\beta$ -CGRP	11.7	0.54	<0.001
BN- $\beta$ -CGRP	9.3	0.50	<0.005
VIP- $\alpha$ -CGRP	6.6	0.40	<0.05

F ratio and p values were determined by ANOVA. r=correlation coefficient. BN=bombesin; EG=enteroglucagon.

plasma enteroglucagon, a gut mucosal peptide, with VIP and  $\beta$ -CGRP is consistent with the proposition that these neuropeptides were also released from the reperfused intestine. Enteroglucagon was probably released by an active process rather than by passive leakage from damaged cells as brief ischaemic intervals cause intracellular changes only without gross mucosal disruption.<sup>24</sup> In addition, passive leakage would have resulted in a rise in plasma peptide YY also. Although these peptides share a similar mucosal distribution,<sup>22-41</sup> peptide YY is not a putative trophic hormone,<sup>31</sup> a role purported for enteroglucagon.<sup>25-26</sup> The rise in plasma enteroglucagon, whose magnitude depended on the duration of the ischaemic interval and therefore the extent of mucosal damage,<sup>24</sup> could be consistent with a trophic role. Mucosal repair begins soon after the onset of reperfusion<sup>45</sup> and entails activation of ornithine decarboxylase.<sup>46</sup> This enzyme is also activated after bowel resection,<sup>47</sup> a state characterised by raised plasma enteroglucagon.<sup>26</sup> The rise in plasma enteroglucagon seen here preceded the reported activation of ornithine decarboxylase that follows ischaemia/reperfusion.<sup>46</sup> It can therefore be speculated that early release of enteroglucagon may trigger activation of ornithine decarboxylase leading to repair of the injured intestinal mucosa.

In conclusion, we have shown a differential response of gut peptides to intestinal ischaemia/reperfusion. The release of potent vasodilators coincides with reactive hyperaemia whereby exposure of ischaemic tissues to oxygenated blood could lead to reperfusion injury. Enteroglucagon, whose release is proportional to the severity of the ischaemic injury, could participate in subsequent mucosal repair. Bombesin may play a pivotal part by facilitating peptide release. The concerted actions of these peptides, unopposed by vasoconstrictors and somatostatin, may be instrumental in producing the characteristic pathophysiological consequences of intestinal ischaemia/reperfusion.

These data were presented in part to the Surgical Research Society and published in abstract form (*Br J Surg* 1988; 75: 1260).

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