

Use of growth-hormone-releasing peptide-6 (GHRP-6) for the prevention of multiple organ failure

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A B S T R A C T

Novel therapies for the treatment of MOF (multiple organ failure) are required. In the present study, we examined the effect of synthetic GHRP-6 (growth hormone-releasing peptide-6) on cell migration and proliferation using rat intestinal epithelial (IEC-6) and human colonic cancer (HT29) cells as *in vitro* models of injury. In addition, we examined its efficacy when given alone and in combination with the potent protective factor EGF (epidermal growth factor) in an *in vivo* model of MOF (using two hepatic vessel ischaemia/reperfusion protocols; 45 min of ischaemia and 45 min of reperfusion or 90 min of ischaemia and 120 min of reperfusion). *In vitro* studies showed that GHRP-6 directly influenced gut epithelial function as its addition caused a 3-fold increase in the rate of cell migration of IEC-6 and HT29 cells ($P < 0.01$), but did not increase proliferation ($[^3\text{H}]$ thymidine incorporation). *In vivo* studies showed that, compared with baseline values, ischaemia/reperfusion caused marked hepatic and intestinal damage (histological scoring), neutrophilic infiltration (myeloperoxidase assay; 5-fold increase) and lipid peroxidation (malondialdehyde assay; 4-fold increase). Pre-treatment with GHRP-6 (120 $\mu\text{g}/\text{kg}$ of body weight, intraperitoneally) alone truncated these effects by 50–85% (all $P < 0.05$) and an additional benefit was seen when GHRP-6 was used in combination with EGF (1 mg/kg of body weight, intraperitoneally). Lung and renal injuries were also reduced by these pre-treatments. In conclusion, administration of GHRP-6, given alone or in combination with EGF to enhance its effects, may provide a novel simple approach for the prevention and treatment of MOF and other injuries of the gastrointestinal tract. In view of these findings, further studies appear justified.

Key words: epidermal growth factor (EGF), growth-hormone-releasing peptide (GHRP), gut injury, ischaemia/reperfusion, multiple organ failure, repair, recombinant peptide.

Abbreviations: ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; FCS, foetal calf serum; GH, growth hormone; GHRP, GH-releasing peptide; i.p., intraperitoneally; I/R, ischaemia/reperfusion; 45 min/45 min I/R, 45 min of ischaemia, followed by 45 min of reperfusion; 90 min/120 min I/R, 90 min of ischaemia, followed by 120 min of reperfusion; MDA, malondialdehyde; MOF, multiple organ failure; MPO, myeloperoxidase; rhEGF, recombinant human EGF; SOD, superoxide dismutase; TGF, transforming growth factor; THP, total hydroperoxides.

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INTRODUCTION

MOF (multiple organ failure) is a severe life-threatening condition that usually occurs as a result of major trauma, burns or fulminant infections. Whatever the initiating event, once established, MOF has a high mortality (up to 80%) [1]. The pathophysiological mechanisms underlying this condition are unclear, although important contributory factors probably include hypoxia, increased intestinal permeability, bacterial translocation, endotoxaemia and uncontrolled systemic inflammatory responses [2].

Several studies suggest that the splanchnic circulation is particularly vulnerable to hypoperfusion, as occurs in low-flow states, such as haemorrhagic shock, and that this hypoperfusion is out of proportion with the overall reduction in cardiac output [3]. Although it is obvious that tissue ischaemia initiates a series of events that can ultimately lead to cellular dysfunction and necrosis, resumption of blood flow can paradoxically create more tissue injury, possibly because of production of oxygen-derived cytotoxic products [4]. The use of I/R (ischaemia/reperfusion) models of injury, therefore, not only have relevance to acute vascular disruption (thrombosis and embolism) and major hepatic surgery, including transplantation, but also to the pathogenesis of development of MOF.

Synthetic and recombinant peptides are being used increasingly for clinical purposes (e.g. human insulin for diabetes and erythropoietin for anaemia of renal failure), but assessment of their value for the treatment of luminal gastroenterological problems is at a much earlier stage [5].

GH (growth hormone) secretagogues compose a group of heterogeneous synthetic peptides and non-peptides that, as well as inducing pituitary GH secretion, also bind to GH secretagogue receptors on peripheral tissues, such as the myocardium, pancreas and bone marrow [6,7]. The physiological role of these peripheral receptors is, however, unclear and the potential value of GHRP (GH-releasing peptide)-6 administration on hepatic and gastrointestinal mucosal integrity is untested.

In this series of studies, we therefore initially examined whether GHRP-6 had potentially useful 'pro-healing' activity using various *in vitro* models of gut injury. Having found positive results, we progressed to test the effect of systemic administration of GHRP-6 in a rat liver I/R model of hepatic injury and MOF. In addition, as we have found previously a beneficial effect of the potent growth factor EGF (epidermal growth factor) when using a related mesenteric I/R model [8], we also examined the results of giving EGF alone and in combination with GHRP-6 (to begin to examine additive/synergistic effects).

MATERIALS AND METHODS

Synthetic and recombinant peptides

GHRP-6 (His-D-Trp-Ala-Trp-D-Phe-Lys-NH₂) was purchased from BCN Peptides. The product in a lyo-

philized form, certified as pyrogen- and contaminant-free, was stored at -20 °C and diluted in sterile saline just prior to its administration.

rhEGF₁₋₅₂ (recombinant human EGF₁₋₅₂), expressed in *Saccharomyces cerevisiae*, was obtained from Heber-Biotec in a lyophilized form. This product consists of a 60:40 mixture of EGF₁₋₅₂ and EGF₁₋₅₁ and is as biologically active as the full length EGF₁₋₅₃ form [9]. Prior to administration, EGF was diluted in 0.9% saline under sterile conditions.

Ethics

Experiments were conducted according to current Local and National regulatory and ethical guidelines.

Study series I: *in vitro* models

Effect of exogenous GHRP-6 on an *in vitro* cell migration model

One of the earliest biological repair responses following injury to tissue cells is the migration of surviving cells over the denuded area caused by the injury to re-establish epithelial integrity. Since it is extremely difficult to study this effect upon tissue inside a human or animal, cell culture models are commonly used as surrogate markers of this pro-migratory response. This method also allows direct actions of the test peptide on the cells to be determined.

Cell migration assays were performed using our methods published previously [10]. Briefly, human colonic carcinoma (HT29) cells or rat intestinal epithelial (IEC6) cells were grown to confluence in six-well plates in DMEM (Dulbecco's modified Eagle's medium) containing 10% (v/v) FCS (foetal calf serum) at 37 °C in 5% CO₂. The monolayers were then wounded by scraping a disposable pipette tip across the dishes, washed with fresh serum-free medium and cultured in serum-free medium in the presence of various test factors. The rate of movement of the anterior edges of the wounded monolayers was then determined by taking serial photomicrographs at various times after wounding [10]. Twenty measurements per field were performed by placing a transparent grid over the photograph and measuring the distance moved from the original wound line. All results are expressed as means ± S.E.M. of three separate experiments.

The various test factors used were GHRP-6 (10–400 µg/ml) and EGF (10 µg/ml; used as a positive control). This dose of EGF was used as we have shown previously [10a] that this stimulates maximal restitution responses in this system. The importance of TGF (transforming growth factor) β in any response seen was analysed by using additional wells which contained GHRP-6 (40 µg/ml) and a TGFβ-neutralizing antibody (100 µg/ml; R&D Systems).

Effect of exogenous GHRP-6 on an *in vitro* cell proliferation model

Cell proliferation assays were performed using our methods published previously [10]. Briefly, HT29 and IEC6 cells were grown in DMEM containing 4 mmol/l glutamine, 10% (v/v) FCS and various test factors. Effects of addition of various doses of GHRP-6 and EGF (10 µg/ml; used as a positive control) were subsequently tested under serum-starved conditions.

To assess the degree of proliferation, [³H]thymidine (2 µCi/well) was included 24 h after the addition of the test factors, and cells were left for a further 24 h. For each condition, the stimulatory or inhibitory effect of the solutions was measured in quadruplicate in six separate wells. Cell viability, determined by the ability to exclude 0.2% Trypan Blue, was always greater than 90%.

Study series 2: *in vivo* model of I/R

Introduction to method

Having shown that GHRP-6 possesses potentially useful biological activity in the *in vitro* systems, we proceeded to examine its effects when used in an *in vivo* hepatic I/R model. Two different timed protocols were used to examine if any effects seen were applicable to both relatively short and more prolonged periods of ischaemia. GHRP-6 was tested alone and also in combination with EGF, as we have shown previously a beneficial effect of EGF in a related mesenteric I/R model [8] and we wanted to determine if any additive/synergistic responses were apparent. The dose of EGF used in the present study (1 mg/kg of body weight) was similar to that used in our study reported previously [8].

Animals

Adult male Wistar rats (200–250 g) were purchased from the National Center for Laboratory Animals and were allowed access to food and water *ad libitum*.

Induction of I/R injury

Animals were anaesthetized with urethane [10 mg/kg of body weight, i.p. (intraperitoneally)] and placed in a supine position on a heating pad in order to maintain body temperature between 36 and 37°C. To induce hepatic ischaemia, a midline laparotomy was used and the blood supply of the right lobe of the liver was interrupted by placing a bulldog clamp (Fine Science Tools) at the level of the hepatic artery and the portal vein branches. Upon completion of the ischaemia time, reperfusion was initiated by removing the clamp. Reflow was confirmed by the macroscopic inspection of the target lobe. No animals were discarded due to non-reflow states. Animals remained anaesthetized throughout the experiment.

Two different I/R time protocols were used: (i) 45 min/45 min I/R (45 min of ischaemia, followed by 45 min of

reperfusion; *n* = 6 per group), and (ii) 90 min/120 min I/R (90 min of ischaemia, followed by 120 min of reperfusion; *n* = 10–12 per group).

Experimental design

For both I/R protocols, rats were randomly assigned to five experimental groups as follows: group 1 (sham ischaemia), animals received saline (placebo; 1 ml, i.p.) and 40 min later underwent all procedures, including laparotomy, liver exposure and manipulation, but the hepatic artery and the portal vein branches were not clamped; group 2 (I/R group), animals received saline (placebo, 1 ml, i.p.) and 40 min later underwent I/R; group 3 (I/R with GHRP-6), animals received GHRP-6 (120 µg/kg of body weight, i.p.) and 40 min later underwent I/R; group 4 (I/R with EGF), animals received rhEGF (1 mg/kg of body weight, i.p.) and 40 min later underwent I/R; group 5 (I/R with GHRP-6 + EGF), animals received GHRP-6 (120 µg/kg of body weight, i.p.) and rhEGF (1 mg/kg of body weight, i.p.) and 40 min later underwent I/R.

Autopsy and sample processing

At the end of the study periods, blood samples were obtained from the abdominal aorta for biochemical determinations. Serum was obtained, aliquoted and stored at –20°C until processing. Rats were subjected to autopsy, and samples of different regions from the right ischaemic lobe were collected for subsequent histopathological examination and tissue homogenization. In addition, representative samples were collected from lungs, kidneys, jejunum and ileum. Samples to be processed for histological study were immediately placed in 10% buffered formalin and subsequently paraffin-embedded and stained with haematoxylin/eosin.

Blood analyses

Serum levels of ALAT (alanine aminotransferase) and ASAT (aspartate aminotransferase), used as markers of hepatocyte injury, were determined using a commercial kit according to the manufacturer's instruction (Sigma). Serum creatinine levels, used as a marker of renal function, were determined using standard colorimetric methods.

Tissue biochemical analyses

The oxidative state of the liver was analysed by measurement of both enzyme activities [SOD (superoxide dismutase) and catalase] and chemical components [THP (total hydroperoxides) and MDA (malondialdehyde) levels]. MDA levels are a commonly used marker of lipid peroxidation. In addition, liver and intestinal MPO (myeloperoxidase) activities were measured as a marker of neutrophilic infiltration.

For liver tissue biochemical studies of MDA, THP and SOD, tissue was homogenized [1:10 (w/v)] in 50 mmol/l

KCl/5 mmol/l histidine buffer (pH 7.4), followed by centrifugation at 5000 g for 20 min at 4 °C. The supernatants were collected, aliquoted and stored at -20 °C until assay. All the biochemical parameters were determined by spectrophotometric methods. MDA content was assessed using the Bioxytech LPO-586 kit (Bio-Rad Laboratories), and THP were determined using the Bioxytech H₂O₂-560 kit (Bio-Rad Laboratories). SOD activity was determined by following changes in autoxidation of pyrogallol in response to adding the homogenate [11]. MPO activity was determined using a modification of the method described by Krawisz et al. [12], and 1 unit of MPO activity was defined as the quantity of enzyme that degrades 1 µmol of H₂O₂/min at 25 °C. Biochemical data were adjusted to reflect total protein concentration using a commercial spectrophotometric protein dye kit (Bio-Rad Laboratories).

Histological assessment

All tissues were assessed in a blinded manner.

Small intestine The total lengths of the small intestine were measured and then split longitudinally to allow a macroscopic assessment of the percentage injured area. The percentage of damage was calculated by measuring (cm) all the regions showing gross macroscopic changes, such as petechiae and haemorrhagic areas, and considering the whole length of the small intestine (in cm) as 100%. Eight equal-spaced 2 cm segments from the length of the small bowel were then collected for histological assessment.

For the microscopic assessment, mucosal damage of the small intestine was quantitatively assessed according to the grading system of Chiu et al. [13]. This system uses a scale of 0–5, where 0 is normal mucosa; 1 is development of subepithelial (Gruenhagen's) spaces; 2 is extension of the subepithelial space with moderate epithelial lifting from the lamina propria; 3 is extensive epithelial lifting with occasional denuded villi tips; 4 is denuded villi with exposed lamina propria and dilated capillaries, and 5 is disintegration of the lamina propria, haemorrhage and ulceration. The mean scores of 30–40 villi from each of the eight segments for each animal were pooled to provide an average score for the intestine of that animal.

Liver For each animal, the degree of liver damage was determined in at least five different lobar regions and graded using the modified Suzuki scoring system [14]. Briefly, the various changes noted are sinusoidal congestion, hepatocyte necrosis and ballooning degeneration. The specimen was then graded from 0–4, where no necrosis or congestion/centrilobular ballooning was given a score of 0, and severe congestion/ballooning degeneration as well as > 60% lobular necrosis was given a value of 4.

Kidney Each sample was classified in a blinded fashion into one of three groups: 0, essentially normal histology;

1, moderate, probably reversible, changes (hydropic cytoplasmic changes); and 2, severe changes (nuclear breakdown or cellular detachment from the tubule basement membrane).

Lungs Lung interstitial damage ranged from normal to showing varying degrees of septal thickening, hypercellularity, neutrophilic recruitment, interstitial adhesion and alveolar luminal reduction. Each sample was classified in a blinded fashion into one of three groups: 0, essentially normal histology; 1, abnormal showing some of the changes described above, and 2, grossly abnormal showing all of the changes described above.

Data analysis

Data were analysed using ANOVA with treatment as factor. Where significant effects were seen on the ANOVA ($P < 0.05$), individual comparisons based on the group mean square error and residual were performed, a method equivalent to multiple comparisons analyses.

RESULTS

Study series 1: *in vitro* studies

Restitution assays

GHRP-6 caused pro-migratory activity of wounded monolayers in both HT29 and IEC6 cells in a dose-dependent manner. Maximal effects were observed at 40 µg/ml for HT29 cells (Figure 1A) and 160 µg/ml for IEC6 cells (Figure 1B).

The addition of a neutralizing anti-TGFβ antibody did not affect the cell migration response caused by GHRP-6 (Figure 1C), suggesting that cell migration in response to GHRP-6 is independent of TGF-β production.

Proliferation assay

GHRP-6 did not induce increased thymidine uptake in HT29 or IEC6 cells at any of the doses tested (Figure 1D).

Study series 2: *in vivo* model of I/R

For both of the short (45 min/45 min I/R)- and longer (90 min/120 min I/R)-timed protocols, the results were essentially the same. The results from the 90 min/120 min I/R protocol are therefore discussed in detail and shown in the Figures and Table 1. The main results from the 45 min/45 min I/R protocol are shown in Table 2 and, in the few instances where the results differ from the 90 min/120 min protocol, these are mentioned in the text.

Liver

Biochemical analyses I/R caused an approx. 10-fold increase in serum ASAT and ALAT. Pre-administration of either GHRP-6 or EGF alone reduced this rise by

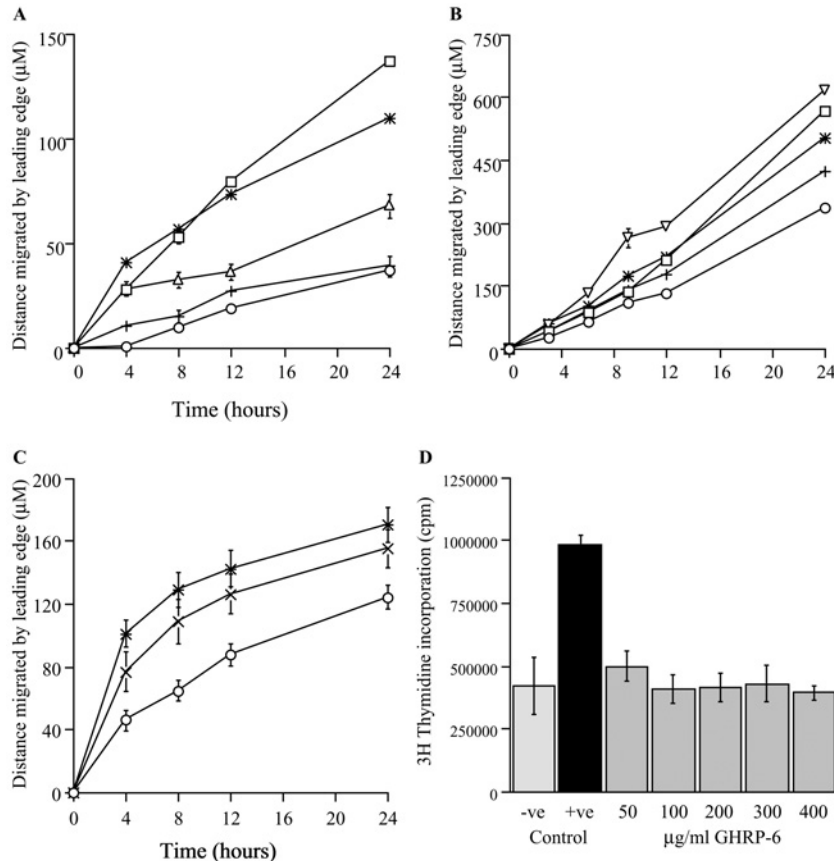


Figure 1 Effect of GHRP-6 on the rate of migration (restitution) or proliferation of various gastrointestinal cell lines

The addition of GHRP-6 to wounded monolayers of (A) HT29 cells or (B) IEC6 cells caused a dose-dependent increase in the rate of migration compared with the negative control (○, no GHRP-6 added). The various doses tested were 1 μg/ml (+), 20 μg/ml (△), 40 μg/ml (*) and 160 μg/ml (▽). Maximum effects were seen at 40 μg/ml in HT29 cells and 160 μg/ml in IEC6 cells. Cells were treated with 10% (v/v) FCS as positive control (□). $P < 0.01$ compared with the negative control at all doses above 1 μg/ml at each time point after 4 h. (C) The pro-migratory effect of GHRP-6 on HT29 cells was not affected by co-incubating with a neutralizing anti-TGFβ antibody. ○, Negative control (no GHRP-6); *, cells incubated with 40 μg/ml GHRP-6; and X, cells incubated with 40 μg/ml GHRP-6 and a neutralizing anti-TGFβ antibody. Similar results were seen using IEC6 cells (results not shown). (D) HT29 cells incubated in DMEM alone (negative control; -ve) had a [³H]thymidine uptake of approx. 400 000 c.p.m. Addition of EGF (10 μg/ml, positive control; +ve) caused an approximate doubling of [³H]thymidine uptake, whereas GHRP-6 (50–400 μg/ml) did not increase [³H]thymidine uptake above baseline. Similar results were seen with IEC6 cells (results not shown).

approx. 50% and combination treatment resulted in a further reduction in enzyme levels (Figure 2 and Table 1).

I/R caused the MDA levels (marker of lipid peroxidation) to increase by approx. 4–5 fold (Figure 2). In the 90 min/120 min protocol, this rise was truncated by approx. 50% in animals that had received GHRP-6 or EGF alone and virtually completely prevented by pre-treatment with GHRP-6 + EGF together (Figure 2). Similar results were seen in animals undergoing the 45 min/45 min I/R protocol, although the rise in MDA was slightly less marked and either peptide given alone was sufficient to prevent an increase in MDA levels (Table 2). Similarly, animals that received placebo and underwent the 90 min/120 min I/R protocol had a 3–4-fold increase in THP (Table 1). GHRP-6 or EGF

alone truncated this response by approx. 75% with combination treatment preventing the rise completely (Table 1). Similar results were seen in animals that underwent the 45 min/45 min I/R protocol, although the amount of THP produced was less (I/R + saline-treated animals having an approx. 2-fold increase above sham-operated animals; Table 2).

I/R caused an approx. 60% fall in hepatic SOD levels, and this change was partially reversed by pre-treatment with either GHRP-6 or EGF alone. A further improvement was seen in animals that had received the combination treatment (Figure 2 and Table 2).

Catalase activity was increased by approx. 30-fold in response to I/R. This increase was markedly truncated in animals that had received either GHRP-6 or EGF

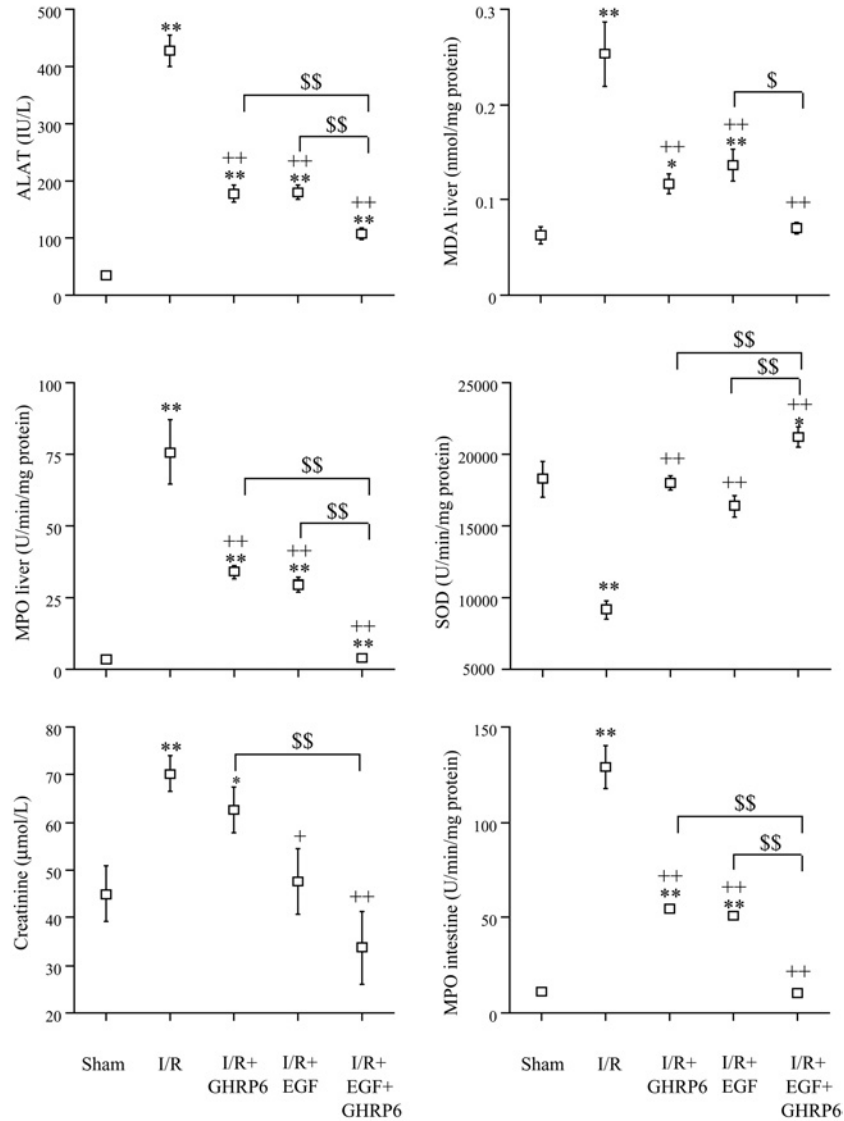


Figure 2 Influence of pre-administration of GHRP-6 and EGF alone or in combination on injury sustained in various organs. Rats (10–12 per group) were pre-treated with GHRP-6 (120 μ g/kg of body weight, i.p.) and EGF (1 mg/ml, i.p.) and then underwent organ injury induced by 90 min of hepatic vessel clamping, followed by 120 min of reperfusion. Animals were then killed and blood and tissue collected for various assays of tissue injury. ALAT is a marker of hepatic injury, MDA is a marker of lipid peroxidation and, along with SOD, allows assessment of the oxidative state of the liver. Liver and intestinal MPO activity was measured as a marker of neutrophilic infiltration. Serum creatinine was used as a marker of renal function. Values are means \pm S.E.M. * P < 0.05 and ** P < 0.01 compared with the equivalent value in sham-operated animals. + P < 0.05 and ++ P < 0.01 compared with the equivalent value in animals treated with I/R + saline. \$ P < 0.05 and \$\$ P < 0.01 when the values in animals given combination therapy (GHRP-6 + EGF) are compared with the values in animals given the same dose of either GHRP-6 or EGF alone.

Table 1 Effect of GHRP-6 and EGF on injury induced by 90 min/120 min of hepatic I/R

Values are means \pm S.E.M., n = 10–12 per group. Also see Figure 1. * P < 0.05 and ** P < 0.01 compared with the equivalent value in sham-operated animals. ++ P < 0.01 compared with the equivalent value in I/R animals. \$ P < 0.05 and \$\$ P < 0.01 when the values in animals given combination therapy (GHRP-6 + EGF) are compared with those in animals given the same dose of either GHRP-6 or EGF alone. IU, international units.

	Sham operation (laparotomy)	I/R	I/R + GHRP-6	I/R + EGF	I/R + GHRP-6 + EGF
ASAT (IU/l)	34 \pm 4	1452 \pm 308**	543 \pm 123***	404 \pm 82**	115 \pm 33**
Catalase (units \cdot min ⁻¹ \cdot mg ⁻¹ of protein)	16 \pm 4	581 \pm 57**	31 \pm 4**	58 \pm 13**	20 \pm 4**
THP (μ mol/mg of protein)	27 \pm 3	109 \pm 16**	51 \pm 2***	43 \pm 2**	21 \pm 2**

Table 2 Effect of GHRP-6 and EGF on injury induced by 45 min/45 min of hepatic I/R

Values are means \pm S.E.M., $n = 6$ for each group. * $P < 0.05$ and ** $P < 0.01$ compared with the equivalent value in sham-operated animals. +++ $P < 0.01$ compared with the equivalent value in I/R animals. \$ $P < 0.05$ and \$\$ $P < 0.01$ when the values seen in animals given combination therapy (GHRP-6 + EGF) are compared with those in animals given the same dose of either GHRP-6 or EGF alone. IU, international units.

	Sham operation (laparotomy)	I/R	I/R + GHRP-6	I/R + EGF	I/R + GHRP-6 + EGF
MPO intestine (units \cdot min ⁻¹ \cdot mg ⁻¹ of protein)	26 \pm 3	148 \pm 13**	60 \pm 8***+\$\$	40 \pm 5+++	16 \pm 2+++
MDA liver (nmol/mg of protein)	0.31 \pm 0.01	1.17 \pm 0.11**	0.37 \pm 0.01+++	0.29 \pm 0.01+++	0.19 \pm 0.01+++
MPO liver (units \cdot min ⁻¹ \cdot mg ⁻¹ of protein)	19 \pm 3	105 \pm 9**	38 \pm 6+++	34 \pm 10+++	8 \pm 1+++
ASAT (IU/l)	13 \pm 2	116 \pm 11**	62 \pm 7***+\$\$	57 \pm 2***+\$\$	33 \pm 4***+
ALAT (IU/l)	21 \pm 3	157 \pm 19**	69 \pm 16***+	82 \pm 13***+	61 \pm 8***+
Catalase (units \cdot min ⁻¹ \cdot mg ⁻¹ of protein)	9 \pm 2	288 \pm 28**	106 \pm 7***+\$\$	63 \pm 2***+	47 \pm 5***+
THP (μ mol/mg of protein)	182 \pm 16	295 \pm 13**	168 \pm 5+++	123 \pm 20***+	108 \pm 8***+
10 ⁻³ \times SOD (units \cdot min ⁻¹ \cdot mg ⁻¹ of protein)	32.5 \pm 1.3	14.2 \pm 1.8**	22.6 \pm 1.0***+\$\$	20.2 \pm 0.5***+\$\$	26.5 \pm 0.9***+
Creatinine (μ mol/l)	43 \pm 5	84 \pm 8**	67 \pm 16	70 \pm 5*	79 \pm 11**

alone (causing a 60–70% reduction), with GHRP-6 + EGF combination treatment truncating this response by approx. 90% (Tables 1 and 2).

Histology Sham-operated animals had an essentially normal liver histology (Figure 3). Animals that had undergone I/R with placebo (saline) injection had severe changes, consisting of areas of necrosis, haemorrhage, cytoplasmic ballooning and sinusoidal distension. Animals that had been pre-treated with GHRP-6 alone, EGF alone or the GHRP-6 + EGF combination therapy all showed improvements compared with the I/R group, with the combination therapy appearing to have the most protective effect (Figure 3). Assessment using the microscopic scoring system confirmed these results; all animals that underwent I/R and received placebo had scores of 3 or 4, whereas six out of ten animals that had received combination therapy had scores of 0 (Figure 4).

Intestine

I/R alone resulted in macroscopically obvious injury affecting 73 \pm 4% of the intestinal length. Pre-treatment with either peptide alone significantly decreased ($P < 0.01$) the degree of macroscopic injury (27 \pm 3 and 30 \pm 2% for GHRP-6- and EGF-treated animals respectively), with the most beneficial effect being seen in animals that had received both GHRP-6 and EGF (19 \pm 2%; $P < 0.01$ compared with I/R alone or I/R plus either peptide given alone).

Histological assessment showed I/R caused severe mucosal damage, with most animals showing complete loss of villous architecture and extensive areas of mucosal infarction (Figure 3). These changes were much less prominent in animals that had received GHRP-6 alone, EGF alone or the GHRP-6 + EGF combination treatment (Figure 3). Quantitative assessment showed similar effects; all animals that underwent I/R and received placebo had scores of 4 or 5, whereas six out

of ten animals that had received combination therapy had scores of 0 (Figure 4).

Kidney

Biochemical analysis In the animals undergoing the 90 min/120 min I/R protocol, serum creatinine levels rose from 45 to 70 μ mol/l in response to I/R. Pre-treatment with GHRP-6 was associated with a 30% (non-significant) fall in creatinine levels, whereas pre-treatment with EGF either alone or in combination with GHRP-6 resulted in the creatinine levels remaining in the normal (sham-operated) range (Figure 2). A similar trend was seen in animals that underwent the 45 min/45 min I/R protocol, although the beneficial effects were less marked and non-significant (Table 2).

Histology Animals that had undergone I/R but not received GHRP-6 or EGF all showed moderate or severe renal injury comprising nuclear breakdown or cellular detachment from the tubule basement membrane. Administration of GHRP-6 or EGF given alone, or in combination, tended to reduce the degree of injury; GHRP-6 + EGF combination treatment had the most beneficial effect with nine out of ten animals having essentially normal renal histology by semi-quantitative scoring (Figure 4).

Lung

Histology Animals that had received I/R without GHRP-6 or EGF had severe changes comprising septal thickening, hypercellularity, neutrophilic recruitment, interstitial adhesion and alveolar luminal reduction (Figure 3). None of the animals that had received I/R without GHRP-6 or EGF had normal lung histology, whereas nine out of ten animals that had received both peptides had normal histology. Animals that had received either peptide alone occupied intermediate positions (Figures 3 and 4).

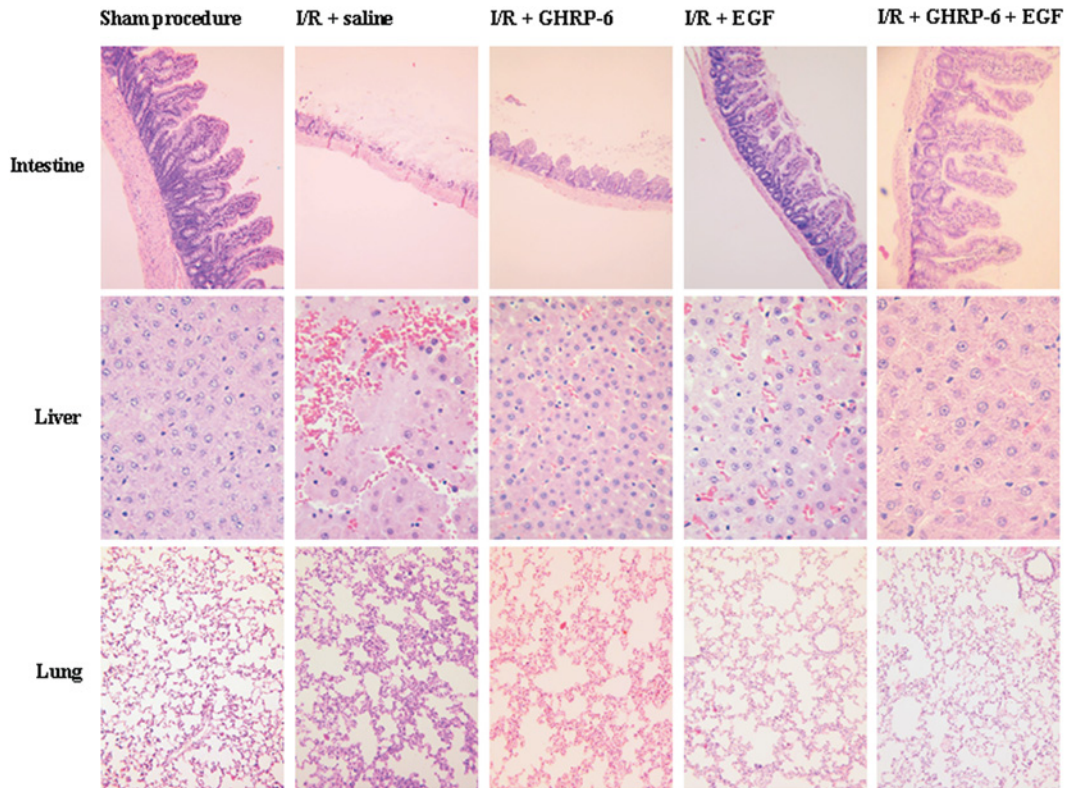


Figure 3 Histopathology of rats given placebo (saline), or GHRP-6 and EGF alone or in combination prior to 90 min hepatic vessel clamping followed by 120 min reperfusion

Compared with sham-operated animals, rats that underwent I/R, but did not receive GHRP-6 or EGF, had severe changes. Administration of either peptide alone improved histological appearances with the most improvement being seen in animals that received the combination of GHRP-6 and EGF. Original magnification of intestine, lungs and liver was $\times 10$, $\times 10$ and $\times 40$ respectively.

DISCUSSION

Using *in vitro* models of injury and repair, we have shown that GHRP-6 stimulates gut epithelial restitution, but not proliferation. *In vivo* studies have shown that pre-administration of GHRP-6 reduced the amount of intestinal and extra-intestinal injury caused by hepatic vessel I/R and that added benefit was observed if EGF was co-administered with GHRP-6.

The control of release of endogenous GH from the pituitary gland is thought to be partially mediated by the presence of GHRP receptors acting via a specific G-protein-coupled receptor pathway, the natural ligand of which is probably the 28-amino-acid peptide ghrelin [15,16]. During the course of research into the control of GH release, several peptides that induce GH secretion were developed and one of the most potent was the hexapeptide GHRP-6 [15,16] used in the present study. Using a variety of GH secretagogue molecules, it is now known that, in addition to being present within the pituitary gland, GHRP receptors are also present in several peripheral tissues, including bone marrow, spleen, pancreas, thyroid and myocardium [6,7], suggesting

additional roles for GHRP ligands that extend beyond GH release.

GHRP-6 stimulated cell migration of the human colonic cell line HT29 and the rat intestinal cell line IEC6, showing that these effects were not species specific and that GHRP-6 was able to influence gut epithelial function by acting directly on the cells. The pro-migratory effects of some of the well established pro-migratory 'growth factors', such as $\text{IFN}\gamma$ (interferon γ), $\text{TGF}\alpha$ and EGF, are dependent upon their ability to induce $\text{TGF}\beta$ release into the medium [17]. It is, therefore, of interest that we found that the pro-migratory activity of GHRP-6 was not blocked by adding a neutralizing anti- $\text{TGF}\beta$ antibody. Caution always has to be shown, however, in extrapolating from the *in vitro* situation (utilizing cancer cell lines) to the *in vivo* situation.

GHRP-6 has been reported previously to stimulate proliferation of the hepatoma cell line HepG2, human pancreatic and prostate cancer cell lines and rat pituitary somatotrophs, possibly acting through the MAPK (mitogen-activated protein kinase) and ERK (extracellular-signal-regulating kinase) pathways [18,19]. In contrast, GHRP-6 possessed anti-proliferative activity

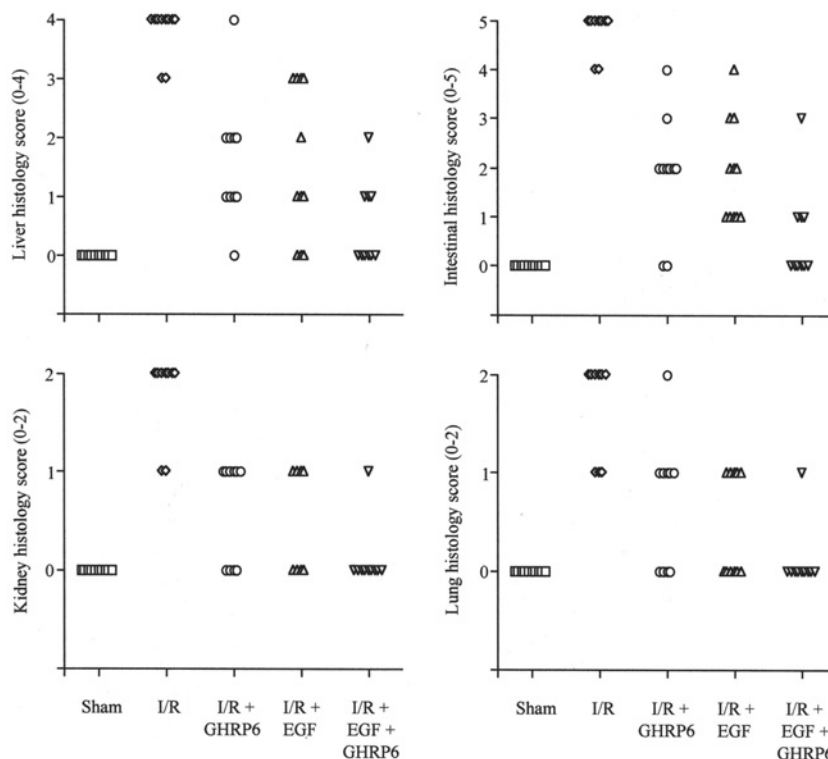


Figure 4 Histomorphometric assessment of histological injury in various organs

Quantitative assessment, using well-validated histological scoring systems, was performed on the livers (modified Suzuki scoring scheme [14]) and intestines (Chiu scoring scheme [13]). In addition, semi-quantitative assessments of lungs and kidneys (scale: 0, normal; and 2, grossly abnormal) were also performed. See text for details of the parameters of assessment.

when added to the human lung cancer cell line CALU-1 [20]. To the best of our knowledge, studies on the effect of GHRP-6 on luminal gut epithelial cells have not been assessed previously. We found that GHRP-6 had no effect on proliferation using either HT29 or IEC6 cells, even though GHRP-6 receptors are presumably present (based on the pro-restitutive activity in the same cells).

The use of arterial occlusion followed by reperfusion is a well-established model of injury resulting from acute vascular occlusion as occurs following embolism or thrombosis. In addition, it is used as a model for loss of the intestinal barrier function associated with haemorrhagic shock, major burns and multiple traumas, which can result in MOF [21]. Several models have been used to mimic the early stages of MOF. I/R has the advantage of being more physiologically relevant than administration of toxic agents, such as thioacetamide [22], as the major factors causing injury are probably internally generated pro-inflammatory cytokines and free radical production [4,23,24], rather than resulting from metabolism of an external damaging agent. Mesenteric artery occlusion is one of the most popular models used (for example, [8]), but suffers from the drawback that much of the intestinal injury is induced directly. The mesenteric I/R model, therefore, although of direct relevance if studies

are being performed in relation to therapies of mesenteric thrombosis, has limitations if therapeutic interventions are being studied in relation to gut changes in MOF, where complete occlusion of the mesenteric vessels usually does not occur. It was because of these issues that we decided to use the liver vessel clamping technique.

I/R caused marked hepatic necrosis as demonstrated by histology and elevated ALAT and ASAT plasma levels. Addition of GHRP-6 markedly truncated the degree of damage determined using all of these parameters. The mechanisms underlying I/R-induced injury and the protective effects of GHRP-6 are likely to be complex and multi-factorial. During hypoxic conditions, there is up-regulation of cell adhesion molecules [25], facilitating recruitment of inflammatory cells to ischaemic areas. Our studies confirmed a marked influx of inflammatory infiltrate within the liver, along with a rise in its associated marker, MPO. Although GHRP-6 has not been directly assessed, administration of ghrelin, the natural receptor ligand homologue of GHRP-6, has been shown to reduce the adhesion of mononuclear cells to endothelial cells activated with TNF α (tumour necrosis factor α) [26]. It is important to note, however, that the influx of inflammatory cells was not restricted to the intestine, but also affected distant organs such as the lungs. This must either

be due to an alteration in circulating factor(s), such as pro-inflammatory cytokines, or to the priming and activation of inflammatory cells (mainly neutrophils) at the hepatic site that subsequently migrate to distant organs.

Production of highly reactive oxygen species and other free-radical-damaging metabolites is known to occur during I/R [23,24,27]. Uncontrolled production of such factors results in cellular damage, including lipid peroxidation, as well as induction of both apoptosis and necrosis [28,29]. We found excessive free radical production in I/R-treated animals, measured indirectly as markedly raised hepatic MDA levels (indicating increased lipid peroxidation) and a general shift in the redox state, as demonstrated by changes in both the enzyme constituents (SOD and catalase) and chemical components (TPH and MDA). The molecular mechanisms underlying the reduction in MDA levels may be due to several factors, including immune modulation. In support of this idea is the finding that ghrelin can directly reduce the pro-inflammatory response of stressed endothelial cells [26], which normally results in a pro-inflammatory cascade and increased free radical production. In addition, GHRP-6 may also have up-regulated the production of cellular antioxidant enzymes. Further studies in this area could potentially measure changes in antioxidant enzyme levels in various hepatic and gastrointestinal cell lines.

GHRP-6 has been shown to reduce the amount of apoptosis in the cerebellar cells of aged rats [30]. The changes seen in our present studies may have been partially mediated by alteration in apoptosis within the liver and other tissues, although the predominant histological feature seen in the liver and intestine was of necrosis. Further investigation into these mechanisms is complex, however, as single cell-culture model systems do not contain inflammatory cells and these are likely to be of major importance in the damaging process *in vivo* (as demonstrated in the present study by raised MPO levels and histology). Similarly, there are major difficulties in attempting to measure the degree of apoptosis within tissues containing large amounts of necrotic tissue. Less damaging models will probably have to be developed to address this question.

Over the last few years, recombinant peptides have been introduced increasingly into the clinical arena (e.g. colony-derived growth factor for bone marrow support and interferon therapy for viral hepatitis). We have examined the effects of EGF in rats undergoing mesenteric I/R previously [8] and also in a clinical trial when administered via enema to patients with colitis [31]. In view of the positive nature of these studies, we also examined and compared the effect of EGF given alone and in combination with GHRP-6 in the present model. We found that EGF given alone was approximately similar in its beneficial effects to those seen with GHRP-6 given alone (although the dose used was 8 times that of GHRP-6). Administration of both peptides together gave additive

or synergistic responses, suggesting that, in the clinical arena, use of multiple therapies may have advantages and deserve further research.

In conclusion, our present studies provide preliminary evidence that the synthetic hexamer GHRP-6 which, because of its small size, is relatively simple and cheap to make may be of benefit for injury associated with visceral vascular hypoperfusion. If patients at high risk of MOF can be identified at an early stage of their admission to hospital, rapid intervention with GHRP-6 may maintain organ viability. Further studies of GHRP-6 given alone, or possibly in combination with EGF to enhance effects, in additional models that allow administration of the peptides after MOF has been induced therefore appear justified.

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